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Poland, Christmas, Sakharov *et al.*

The scientific community cannot complain that some of its members live in states whose laws are illiberal, but should it acquiesce in the arbitrary loss of jobs?

The coincidence of recent events in Eastern Europe with the Christian celebration of the ancient festival of the winter solstice is not accidental. If, in Poland, the tide of social change that has occupied the past eighteen months is to be turned back by the threat of force, when better than this time of the year, when life is hard and food even more scarce than usual? But this is also the time when the despair of the despairing is heightened by the sense that winter's grasp has yet fully to close. This may help explain why a group of scientists in the Soviet Union has written to *Nature* to complain at their government's emigration policy (see page 688). Their letter is unlikely to make life easier for them, at least in the immediate future. The authors are nevertheless aware of what the consequences may be. That they have written their letter is a sign that they consider more conventional means of pressing their case have run into the sand. Will what they have to say be heard against the din of the forced jollity of the days ahead?

The authors of the letter appeal to the international scientific community, of which they wish to be a part. What response is appropriate? This question has much in common with those which have exercised scientific academies in the past several years. A few simple truths stand out. No part of the scientific community is competent to complain at the legal practices of individual governments, however illiberal or unjust. If, for example, it is still the practice somewhere to punish a thief by cutting off his right hand, that is no part of the scientific community's business. Similarly, scientists used to Western ways, and in particular to the knowledge that people's freedom to live and work elsewhere is constrained only by the willingness of host countries to receive them, have no right as scientists to complain that Soviet law gives the administration the right to withhold exit visas. It is true that the scientific community is impoverished – and the Soviet Union made to look foolish – when Soviet scientists are prevented from attending international conferences, but even that is not an occasion for public protest but a matter for diplomacy among the scientific academies (which, nevertheless, should be more energetic in this cause).

The disturbing feature of this week's letter from Moscow is that it suggests that individuals scientists in the Soviet Union may be victimized as scientists while acting within the law. It is common ground that to apply for an exit visa is not a crime. The fact that Dikii *et al.* are Jewish is (or should be) irrelevant, embarrassing though the flood of would-be emigrants to Israel has been for the Soviet government in the past decade. And their plight is by no means novel. Many Soviet citizens who have become in some sense awkward customers have found the loss of their jobs to be the first sign of having given offence and often a prelude to more serious trouble. Moreover, the loss of a job is not exclusively an accident befalling those who wish to go to Israel. There is at least one unpublished case of a graduate student who has been dismissed from the institute at which he was hoping to complete the work for his doctoral thesis on the heels of his application to join his wife in Western Europe. On the face of things, however, even this accumulating evidence of illiberality is not a sufficient cause for the scientific community to take up cudgels. If the convention is being established that those who legally fail to toe the conventional line will in the first instance lose their jobs, and if the sanction applies to all and sundry, surely the international scientific community has no special status in the matter? That is the

simplest (and most convenient) conclusion. It is, however, wrong.

First, it is more than probable that the convention that those who make trouble should lose their jobs will bear with especial severity on scientists in the Soviet Union. Continuity, as Dikii *et al.* proclaim, is crucial, especially early in a career; other kinds of professional people are usually (but not always) less vulnerable. Second, the importance (numerically and prestigiously) attached in the Soviet Union to professional work in science and technology is almost a guarantee that technical people will be prominent among the casualties of illiberality. Third, if scientists lose not only their jobs but access to scientific publication and are at the same time stripped of their degrees – a fanciful process in itself – the record of research is falsified. These are in themselves sufficient reasons why the scientific community elsewhere should take more than a passing interest in what is happening in the Soviet Union. But the most serious cause for alarm is the now well-understood procedure for arranging that those who figuratively blot their copybooks will then lose their jobs. Most Soviet laboratories have two directors, the better known of whom is a distinguished scientist. His partner is more directly concerned with political matters, including the hiring and firing of people. One of the unacceptable ironies of these arrangements is that the scientific heads of important institutions (who seem to have no difficulty with short-stay exit visas) are able to boast of the bright young people who work for them but to deny responsibility for those who have lost their jobs, to become dependent on the charity of friends and former colleagues. Such detachment (even if occasionally mirrored in the attitudes of the heads of laboratories in the West) is unfortunately not merely offensive but potentially destructive of the scientific enterprise. If bright young people are arbitrarily denied the chance to work the stint of which they are capable, and if the numbers are as substantial as those now accumulating in the Soviet Union, the enterprise will be undermined, not just impoverished. Those who enjoy the privilege of being directors of important Soviet institutes should be left in no doubt of how, by their indifference to or neglect of what is happening to the people in their charge, the consequence will be not merely to impoverish but to corrupt the scientific enterprise.

None of this will help Dikii *et al.* What should they do? Go on hunger-strike, like Andrei and Yelena Sakharov? For, contrary to expectations (see *Nature* 26 November, p.295) that ploy has worked. Dr Sakharov's putative daughter-in-law, Liza Alexeyeva, has now joined his stepson in the United States. Mercifully, the Sakharovs are able to eat again (but they must please remember that they cannot keep on doing this, as they get older, with like impunity). Time will no doubt tell on what basis Liza Alexeyeva's departure from her homeland and the fasting Sakharovs was arranged. The Sakharovs probably compromised very little. But it is also plain that their despairing wager of their lives has been an uncharacteristically narrow venture. Their daughter-in-law has got away. The graduate student who impetuously married his legal wife is still stuck without a job somewhere in the Soviet Union. Should he ask his parents to go on hunger-strike? Both he and they, of course, know that that would carry relatively little weight. They may all think it a pity that the Sakharovs have spent their great influence on the solution of one person's problem when they might have been able to contribute to



a much more general resolution of a general important problem — even if one that is not directly the concern of the scientific community. That even brave people such as the Sakharovs might have been able to accomplish a step towards that goal is naturally a conjecture — and at times like the winter solstice, even people like them will be forgiven for putting Christmassy personal considerations before grand and perhaps unattainable causes.

None of this will much help the scientific academies or the individual members of the scientific community to know more clearly where they should stand on the events of the past few weeks. On the most immediate issues, they have no competence to make pronouncements of a scientific character even if they may choose to be vociferous in other roles. Precisely how the residue of what the Poles used to call Solidarity should be succoured is beyond their competence as scientists (although some, as scientists, may in future think differently of the problems of European defence). The only legitimate opening in this haunting argument for the scientific community is that represented by the hypocrisy of the institute directors from the East, who pretend that all the dreadful things that happen to those who used to be their young men and women are outside their competence. That defence can no longer wash — and it would be better for all concerned if the Soviet authorities would make (as would be their right) a simple rule that those who have benefited from the Soviet educational system must work their passage towards emigration by some system of indenture — so many years of public service for such and such a qualification would at least let people know where they stand, and would be consistent with the principle that sovereign states should be free to decide how to run their affairs. The growing practice of depriving skilled people as a kind of punishment of the right to practise their skills is a loss of more than a Soviet resource, and is offensive. Indeed, it is a disgrace.

British academic agony

The British government has relaxed pressure on its universities, which may fail to take advantage.

The British government has now agreed to pay the second instalment of the cost of reorganizing and running down the British university system, decreed by the then Secretary of State for Education and Science just over a year ago. During the present academic year (which ends on 31 July 1982), the University Grants Committee has been able to set aside a total of £20 million to help with the cost of paying off redundant academics. In the following year, there will be a further £50 million to spend on measures of reorganization in the university system, but the government bravely says that it hopes not all of that will have to be spent on putting academics out to grass. If the Committee of Vice-Chancellors is to be taken at its first word on the subject (which would be imprudent), the settlement for next year will be totally inadequate to pension off university teachers whose tenure of their jobs cannot be sustained. In reality, of course, the crunch will come in the succeeding financial year, when the universities will have reduced their student numbers to those required of them by the University Grants Committee, and when their urgent need will be to trim their sails for the long haul that will by then seem to lie ahead. But the government has promised to provide a still unspecified amount of money to cover the costs of that year's mayhem; is it hoping that in the meantime (during 1982–83) the universities will have the wit to think of doing novel things, things they have not thought of yet?

The difficulty in understanding the conflict between the British government and the British university system is that it is not so much a conflict between an irresistible projectile and an immovable object as between blancmange and jelly (or jello). The government has discovered, late in the day, that the cost of firing tenured academics is not negligible, but dare not say so. The universities, on the other hand, are so frightened and affronted by what has happened to them in the past year, in particular by the discovery that they have fewer friends than students, that they

behave as if they have no choice but to pass on to their paymasters the information that they have gathered from the Association of University Teachers, the mild-mannered labour union capable nevertheless of making a lot of noise, that the cost of any change is bound to be greater than the cost of comfortable no-change. The time has come to ask whether even that seemingly prudent course can be wise.

Like the constituents of other university systems, British universities are naturally jealous of their autonomy. Their jealousy is, however, undimmed even while (as now) they are learning to live with instructions from the University Grants Committee that the numbers of students recruited in various fields should be determined in advance. The conventional statement of academic freedom is that self-governing universities should be free to decide for themselves who should teach what to whom. The difficulty, in present circumstances, is that the who is defined by the survivors now in post, that the what is largely determined by extension and that the to whom, the students, make their own numbers through the medium of the Universities Central Council on Admissions, falling so to speak where the academics lie. The new restrictions on student numbers rob the university system of a further degree of freedom, a loss which collectively the universities have not yet chosen to complain about. They should.

Individually if not collectively, they should also give some serious thought to what the more distant future holds. Although the government has been able to put some kind of limit on the cost of other kinds of higher education than those provided by universities, the lack of coherence between the universities and the polytechnics persists as a weakness of both systems. The universities, the more senior of the partners, should acknowledge that it is, in these hard times, for them to bridge the gap. While they are about it, they should also give more serious thought than has been their habit to the needs of their students and of their students' potential employers. British students are too highly specialized; British graduates are too uniformly academic. Not all but most universities should change their habits of selection and their ways of teaching so as to cater for a greater diversity of people and of interests. Over the past twenty years, British universities (singly and collectively) have resisted the notion that students are not all ideally the same, and that students' ambitions sometimes encompass secularly non-academic goals. That is obscurantist.

The plain truth about the distant future is that British universities are at present organized in such a way that they will all (except Oxbridge) sink together unless they have the courage to acknowledge that survival requires that they should exercise the autonomy of which they boast. Several corollaries arise. Academics should not always be paid the same, under "nationally negotiated agreements"; instead, universities should be free to pay their people what they can afford (and earn). Tenure should be limited, taking some account of the need to preserve people's freedom to write (and even to say) what they think fit, but acknowledging that universities cannot make lifelong contracts with their employees while living from hand to mouth themselves. The result of such an erosion of the common academic sense of security would send the better academics flocking to two contrasting kinds of places — secure universities valued for their sheer academic excellence and much more specialized places valued for their special skills at teaching students what much of the real world expects of them. The result, of course, would be more diversity among institutions of higher education. Would that necessarily be disastrous? The system as it is is calamitously uniform.

So how to get from here to there? The interpretation of the British government's budget restrictions by the University Grants Committee will, if unchanged, have an ossifying effect. Universities individually will preserve their present character but will shrink. Strange though it may seem, the universities probably find that dismal prospect preferable to the better alternative — a framework in which they would be enabled but also compelled to find for themselves a niche in the market in higher education.

Balance of US energy research attacked

Advisory group gives comfort to Reagan's foes

Washington

A top level advisory committee to the US Department of Energy (DoE) has told the Reagan Administration politely but firmly that it is not making the best use of its resources for energy research. In particular, the committee says that there is insufficient support for conservation technologies and that too much is being spent on nuclear energy.

The report was published shortly before President Reagan's announcement last week that he is soon to submit plans to Congress for dismantling DoE. He said that the purpose of the move is to create "a strong federal effort in basic research in energy that avoids excessive regulation".

The centrepiece of the reorganization is a new Energy Research and Technology Administration within the Department of Commerce. The plan is expected to meet fierce opposition in Congress, particularly from those who favour an independent energy research agency on the grounds that close links with the Commerce Department would reinforce the alleged pro-nuclear bias.

The criticisms of present energy research policies came from DoE's Energy Research Advisory Board (ERAB), an independent, non-partisan group. The chairman of the board is Mr Louis H. Roddis Jr, previously president of Consolidated Edison of New York, and the report was prepared at short notice at the request of DoE by a panel, led by Dr John S. Foster, vice-president for science and technology at TRW Inc., comprising all ERAB members and several outside consultants.

Objections to the Reagan plan are already coming from another direction, namely congressmen unhappy at the proposal that the nuclear weapons research carried out at the nation's weapons laboratories should be among the activities transferred to the Department of Commerce. Speaking at the National Press Club in Washington last week, commerce secretary S. Malcolm Baldrige said that his department's experience with other research agencies, such as the National Oceanic and Atmospheric Administration, made it a natural place for locating energy research. He also admitted that the Commerce Department's responsibility to assist in increasing exports could logically include the international promotion of American nuclear technology.

The ERAB report takes as its frame of reference the policy guidelines laid down

by the Reagan Administration, thereby insulating itself from the criticism that it is making a political attack on Mr Reagan's policies. Although in principle enthusiastic about the new policy directions, the ERAB committee is concerned about how decisions, such as the cutting back of research funds for conservation and solar technology, have turned out in practice. And the report champions higher energy prices as the best way of encouraging efficient use of energy and utilization of new energy sources.

All 20 members of the committee agreed on the need to emphasize new energy technologies where research has already reached the stage at which the results could, if desired, be directly taken over by the private sector, and should otherwise be dropped if this desire does not exist. For example, the report recommends the elimination of support for research into small-scale hydropower and magnetohydrodynamics (decisions already made by the Reagan Administration), and a sharp reduction in support for research on electric vehicles.

Little change is advocated for most of

the basic sciences whose budgets are the current responsibility of the department, including high energy physics and nuclear physics, and which would be incorporated directly into the new agency. And the board favours greater support for efforts aimed at improving the quality and quantity of scientific personnel at US universities in energy-related areas, described as an area "appropriate for federal concern" but currently receiving only \$10.6 million a year.

The most controversial parts of the report are those which address directly the government's role in supporting research into the generation of electricity through nuclear power. The ERAB report describes the Clinch River fast breeder reactor as being "not an urgent priority", recommending that, under current budget constraints, such a demonstration project should be postponed. Four members of the advisory board, however, dissent from this opinion on the grounds that not proceeding with the construction phase of the fast reactor would mean writing off the \$1 billion that has been invested so far, and

What case for building Clinch River?

Washington

Following Congress's decision last month to give the go-ahead for the construction of the liquid metal fast breeder reactor at Clinch River in Tennessee, the focus of the controversy surrounding the project has shifted back to the Nuclear Regulatory Commission (NRC), which would have to issue a permit allowing the construction to proceed.

Last week, the five members of the commission agreed to consider a request from the Department of Energy that it be given "emergency" exemption from conventional licensing procedures. According to Energy Secretary James Edwards, such an exemption is necessary to avoid the "undue hardship" which, he told NRC, would result from further hold-up in the long-delayed construction plans.

Critics of the reactor, however, claim that the Department of Energy is trying to manoeuvre NRC into providing a provisional construction licence as soon as possible so as to pre-empt further attempts by Congress to terminate the project. The critics claim that their case is substantiated by an internal Department of Energy memorandum, released last week by the Natural Resources Defense Council (NRDC), in which Under-Secretary of Energy Guy Fiske suggests that the request for an emergency exemption from NRC be withheld until the 1982 appropriation bill is passed — an amendment could

have been added forbidding the Department of Energy from making such a request — and that the Council on Environment Quality be asked about securing "strong support" for an internal environmental report establishing the "negligible environmental impact" of the construction of the reactor.

NRDC attorney Barbara Finamore said that the memo "reveals a calculated effort by the Department of Energy to undermine the integrity of the NRC and its licensing process". Supporters of the fast reactor, that the Clinch River project is needed to demonstrate this technology in the US licensing and safety environment.

Although the construction of the Clinch River reactor has been approved by Congress, its completion is not certain. It is generally accepted in Washington that the main reason for Congress's approval was political — the project is strongly backed by Tennessee Senator Howard Baker Jr, the leader of the Senate.

One possible outcome is that preliminary construction work will be carried out, partly to satisfy the pro-nuclear supporters in Congress to whom the fast reactor has become an important symbol, but that eventually even the utility companies which now back the project will agree that it is inappropriate to continue, and the Clinch River reactor will be abandoned on the grounds of its inefficiency and antiquity.

David Dickson

dissipate the "hard-won technology capability" already developed in the United States. The dissenters were ERAB chairman Louis Roddis, William S. Lee, president of Duke Power Company, Roland W. Schmitt, vice-president for corporate research at General Electric and John W. Simpson, a consultant previously with Westinghouse Electric. Described by critics as the "electric mafia" on ERAB, these same four are said to have been successful in toning down parts of the report which criticized the emphasis being given by the Reagan Administration to nuclear research as disproportionate to nuclear's role as an energy source.

The final report states merely that "although no correct balance among energy forms and resources can be defined *a priori*, research and development for electric supply technology is receiving a larger proportion of funding than the present and projected share of electricity in our national energy supplies". In addition to recommending that the Clinch River fast reactor should not be built, the panel suggests that high priority be given to research into nuclear waste disposal and into conventional light-water reactors, and a "little less" funding into both breeder reactor fuel cycle research and magnetic fusion.

David Dickson

Data falsification Harvard acts

Washington

Harvard Medical School announced last week that it was setting up a special committee of both faculty members and outside academics to recommend how the school should deal with future cases in which research workers are accused of producing falsified research data.

This announcement follows the resignation of a member of the medical school research staff, Dr John R. Darsee, who admitted that he had fabricated research data during an experiment last year which involved efforts to limit the damage caused by heart attacks in animals.

Dr Darsee also resigned his post as a research fellow at the Brigham and Women's Hospital in Boston. Before his resignation, according to reported comments from other members of the hospital staff, he had been under consideration to head a hospital laboratory.

As a result of allegations about Dr Darsee's work made last year by other research workers at the medical school, an investigation was carried out, in the course of which Dr Darsee admitted that he had falsified the research results in question.

A statement issued by the dean of the medical school, Dr Daniel C. Tosteson, last week stated that "none of the work which could not be verified has been presented to the scientific community". Earlier this year, Dr Darsee's supervisor, Dr Eugene Braunwald, decided that all

Christmas present for Heidelberg laboratory

The European Molecular Biology Laboratory at Heidelberg has half the Christmas present it was promised a few months ago: a liquid-helium temperature transmission electron microscope lens from Siemens AG, Munich, but not the pictures that should follow from it.

Dr Arthur Jones, head of the electron microscopy group at the laboratory, had hoped the system would have been operational by Christmas. But the lens — only the second commercial such lens in existence (the other went to Berlin) — arrived only a week ago. The first pictures, probably of hydrated crystalline specimens at 4 K, are likely to follow in January. Biological specimens should be in view in the spring.

Excitement is intense in the group about what they see as the most important development in electron microscopy for more than a decade. The Siemens group, headed by Dr I. Dietrich, appears to have shown that specimen damage by the electron beam — which limits the available resolution of a microscope on sensitive, non-periodic biological specimens — may be reduced by orders of magnitude at 4 K.

And at the same time, Dr Jacques Dubochet, in charge of specimen preparation, claims now to produce hydrated biological specimens containing vitrified — non-crystalline — ice. (The formation of crystals would create artefacts and interfere with the imaging of specimens.)

At first, however, the resolution may be no better — or even worse — than with conventional electron microscopy, because the contrast available in hydrated specimens is much less than that possible with negative staining. The reduced beam damage, however, should compensate for this by allowing greater illumination, with the result that the microscope might reveal structures without the artefacts of the conventional stains. Ultimately the use of samples labelled with heavy atoms could make a greater, artefact-free resolution available says Dubochet.

After this "cryo-TEM" will come the scanning version, the "cryo-STEM". The first electron beam down the cryo-STEM is expected by mid-year, with the first images shortly after.

Robert Walgate

abstracts of Dr Darsee's work should be withheld from presentation at the annual meeting of the American Heart Association.

The National Institutes of Health (NIH) were also notified of the alleged falsification of data, since Dr Darsee was working on NIH-sponsored research and was in receipt of an NIH research fellowship, which he has since resigned together with his academic posts.

The advisory panel set up by Dean Tosteson is being chaired by Dr Richard Ross, dean of Johns Hopkins School of Medicine. According to last week's statement, the panel has been asked "to review the case in question, and to indicate whether or not any additional measures should be undertaken, and to recommend procedures for dealing with episodes of this kind in the future".

The committee has already started work, and its report is expected early in 1982.

David Dickson

Molecular biology

Limited progress

When the director-designate of the European Molecular Biology Laboratory (EMBL) in Heidelberg takes over from Sir John Kendrew in April, he will not find much room for movement in the budget. Last June, Sir John asked the ten-nation council for DM 32 million (£7.5 million) for 1982. In the event the council has now agreed to spend DM 30.2 million, roughly a 10 per cent increase on 1981 compared with a German inflation rate of about 6 per cent.

That allows for a small increase in staff this year — some 20–25 of whom 8–10 will be scientists, according to finance director Eckhart Weis. Thus the laboratory will edge ahead of local inflation, but will come nowhere near the "indicative scheme" prepared in 1980 which foresaw a budget increase of 20 per cent and a staff of 265 rather than the 220 now employed. The directorate, for "scientific reasons", did not fill the 265 posts which were available in 1980, and since then the recession has meant that the council would not agree to the budget which would be required to re-offer them.

Meanwhile, the new EMBL director, Professor Lennard Philipson of the University of Uppsala, refuses to define his policy for the laboratory until after he is in post. But he has been taking regular soundings, and, it seems, gaining everyone's confidence. He is said to recognize the important and unusual role that physicists play at the laboratory and the significance of the EMBL outstations at DESY, Hamburg (a synchrotron radiation source which has a long queue of applicants for beam time) and the Institut Laue Langevin, Grenoble (a neutron source).

It seems likely that the new regime will see a greater integration of the work at Heidelberg, and between Heidelberg and the outstations, with the selection of two or three principal areas of biology (cell membranes, for example) as broad foci of interest. But there will be nothing so block-busting as an attack on the whole human chromosome — "that's factory work" said one senior EMBL scientist. On the

instrumentation side, certain techniques, such as gel electrophoresis, which are central to the new methods of molecular biology, may be singled out for increased attention; but Philipson is also said to have shown his support for the great efforts being made at Heidelberg to improve electron microscopy through cooling the microscope and specimen (see accompanying box).

At present Sir John Kendrew is still firmly in charge. He foresees an increase in the number of permanent scientific appointments (still less than a handful) at the laboratory in the next couple of years, as a batch of the early short-term contracts come to an end. Philipson, however, has made no commitments. As for Sir John himself, he feels that the seven years and three months for which he will have held the directorship is quite long enough.

Robert Walgate

Polish crisis

Science in limbo

The "state of war" proclaimed by General Jaruzelski's "Military Council for National Salvation" has brought all academic life in Poland to a standstill. The universities are closed as are several institutes of the academy of sciences. Scores of scientists and scholars have been arrested although some, such as the president of the academy, Dr Aleksander Gieysztor, have since been released. Others escaped arrest only by chance, being away from home when the police came, and are now in hiding. Solidarity organizers in the learned professions and activists from the Independent Students' Association have been interned. The Minister of Science, Higher Education and Technology, Dr Jerzy Nawrocki, is reported to have resigned.

This clamp-down on intellectuals is in sharp contrast with Jaruzelski's previous attitude. In a recent interview, Dr Leonard Sesnewski, one of the vice-presidents of the academy, said that Jaruzelski had always expressed warm feelings for scientists and that, on taking office as prime minister last February, he had immediately visited the academy to ask for their help in getting Poland out of its economic crisis.

Nevertheless, the growing drive for academic autonomy must have caused a certain friction between the general and the academy. At present, the academic secretary of the academy holds quasi-ministerial rank, and is responsible to the prime minister. In recent months, however, there has been considerable pressure from academy members and employees to incorporate into the proposed new bill on the academy clauses that would terminate this structure and make the academic secretary responsible only to his fellow-academicians. There has also been a drive — reflected in the Solidarity Congress resolution on learning

and culture — to break down the barriers between the universities and the academy, so that academy employees could deliver lectures to undergraduates. This trend may have seemed dangerous to the authorities; a number of scholars, expelled or excluded from teaching posts for their political views over the past few years, have found a safe haven in academy research posts insulated from teaching.

For the time being, the concern of scholars abroad is focused on the plight of their Polish colleagues interned or in hiding. Professor Olof Tandberg and his colleagues from the Swedish Academy of Sciences (which traditionally has strong links with Poland) and several members of the Norwegian Academy have launched an appeal for financial support for the families of interned academics. Professor Tandberg has also received an appeal from a group of academy scholars in hiding asking for food, blankets and other essential supplies, including blood plasma. He has called on all other academies throughout the world to join the Norwegian and Swedish academics in this work.

Vera Rich

UK agricultural research

Endangered duo

Two British agricultural research institutes, the Long Ashton Research Station in Bristol and the Animal Breeding Research Organisation in Edinburgh, could be drastically cut or even closed if an economy proposal by the Agricultural Research Council is approved at its next meeting in February. Under threat are all research programmes at the Edinburgh institute except work on fundamental animal genetics, and the Pomology and Food and Beverages Division at Long Ashton.

The council (ARC) hopes the closures will save about £3 million in annual expenditure by 1983–84. (The budget for 1981–82 is £86 million.) According to Dr Ralph Riley, the secretary of ARC, the savings are needed both as a hedge against future government cuts and to allow greater flexibility in supporting research of high priority. The council's research programme has already suffered, he says, from a budget that has not kept up with inflation. Non-payroll expenditure has already been pared to the bone. But precisely how much of the £3 million saved will be available for starting new research projects after cuts in income are taken into account remains uncertain.

If the proposal is approved, the Long Ashton Research Station will be reduced to about one half of its present strength, with the loss of about 100 jobs. The Animal Breeding Research Organisation, however, will probably be closed and the research on animal genetics, which accounts for about one-fifth of the organization's activity, moved elsewhere.

ARC has made the proposal without

consulting the staff or directors of the institutes concerned, who first learnt of it only last week. The idea, according to Dr Riley, was for ARC to propose firm suggestions for savings and then to give those concerned two months in which to make a case before a final decision. The way in which the proposal was arrived at remains something of a mystery, but the main criterion seems to have been an assessment of priorities based on recent reviews of the work of all the council's institutes. Areas in which the council is likely to want to spend more money include fundamental genetics and biotechnology.

If approved, the proposal could have implications for the Ministry of Agriculture, Fisheries and Food, which spends about £44 million a year in ARC institutes on commissioned research. The ministry is optimistic that the work it commissions in the institutes under threat could be transferred to other institutes. Dr Riley, however, believes that this will be possible for most commissioned research but that there may be some areas where the ministry will have to look elsewhere. **Judy Redfearn**

High-energy physics

LEP marches on

The large electron-positron ring is on the move — in more than one sense. LEP will be the next big accelerator at CERN, the European Centre for Nuclear Physics, Geneva, and last week CERN member states finally agreed on the budget under which it will be built. Thus construction can begin as soon as local environmental approval is granted — by January or February, it is hoped. The second move is physical: LEP is to be built in a significantly new position, on a slope, which allows it to slide out of some tricky geological and political problems.

The budget agreed is sufficient, says CERN director-general Professor Herwig Schopper, to build LEP on the schedule foreseen a few months ago: fast enough to give colliding electron and positron beams of sufficiently high energy to provide physicists with copious neutral intermediate vector bosons by the end of 1987.

LEP is expected to cost SF910 million (about £263 million) at 1981 prices, with a further SF40 million (making a total of £275 million) for the first experiments. The money will be drawn from a guaranteed CERN annual budget of SF617 million.

The only major argument at the council was over what inflation index to apply to the 1981 budget to scale it up to 1982 prices. The usual CERN formula, appropriately weighted for CERN's Swiss salaries and expenditure abroad, gave 5.7 per cent. The council quibbled, and agreed on 4.4 per cent, losing CERN over £2 million next year.

The new slope for LEP (it was to have been horizontal) will be about 1.5 per cent from the Jura Mountains in the north west, towards Geneva. This enables the machine

to be slipped away from the cavernous Jura limestone, and further into the stable sandstone of the valley, in which all previous CERN accelerators have been built.

The result is that only 3 km (as opposed to 8 km previously) of the 27 km tunnel for LEP will be in the unpredictable limestone. Moreover, at the Jura side the ring will be higher — about 140 metres below the surface — so that if any difficult water-filled caves are encountered, it will be possible to deal with them from above.

A further — and far from negligible — benefit may prove to be political. There has previously been vociferous environmental opposition to LEP from the French side (the Jura; LEP straddles the French-Swiss border). This opposition rested in large part on the (distributed) effect that the limestone borings might have on the source and flow of the River Allondon, which supplies water to a number of villages. The source was to have been within the LEP ring; now it is outside, and where the ring passes under the river it is within stable sandstone.

LEP will be paid for out of the current budget only by running down certain existing facilities. The intersecting storage rings for protons and other nuclei will be closed at the end of 1983; and operations on the 600-MeV synchrocyclotron (CERN's first accelerator) will be reduced to the mainly Scandinavian Isotope Separator On-Line (ISOLDE), which performs unique experiments on short-lived nuclei and, incidentally, keeps the Nordic countries happy. **Robert Walgate**

UK university funding

No reprieve

The British government is sticking to its guns over cuts in university grants, indicating that universities have failed to convince the government that spreading the cuts over five instead of three years would cost less because compulsory redundancies, involving large compensation payments, would be avoided. Last week, Sir Keith Joseph, Secretary of State for Education and Science, reaffirmed his belief that the cost to the taxpayer will be less if the cuts are implemented quickly.

Hence, it is no surprise that the universities' recurrent grant for 1981–82 has been set broadly in line with the government's expenditure plans of last April. The grant at £995 million will be only £16 million more than the estimate, to account mainly for an inflation rate higher than expected. The research councils, however, may have come off slightly better than feared. At £478 million, the science vote for 1982–83 will be roughly in line with this year's figure, although the allowance for increases during the year is only 5.5 per cent.

Despite his rejection of the universities' argument, Sir Keith has nevertheless accepted that the cost of redundancies

cannot be met out of the universities' recurrent grant. But his allocation of £50 million in 1982–83 for restructuring the university system has already met with derision from the Committee of Vice-Chancellors and Principals which says that the sum is far too small. The vice-chancellors also fear that if the 5.5 per cent inflation allowance cannot be met then the number of redundancies will increase. A sum for restructuring in 1983–84 will be announced some time next year.

Sir Keith expects to reach a decision early in the new year on the scheme proposed by the vice-chancellors' committee for compensating redundant academics. Academics who take their cases to court, however, could be awarded considerably more than indicated under the scheme, making it almost impossible at present to estimate the total redundancy bill.

Advanced higher education has fared worse than any other sector of education under the government's cutbacks. The overall reduction in the education budget next year compared with this is one per cent, with further education for non-academic school leavers winning increased support. Clearly, the universities have lost out to the much stronger voice of the growing numbers of unemployed.

Judy Redfearn

Recombinant DNA research EEC safety dispute

Brussels

A deep division of opinion has become evident among the EEC's institutions over the need for strict legal controls to minimize the dangers of research using recombinant DNA techniques. By a narrow margin, the European Parliament's Committee on the Environment, Public Health and Consumer Protection came out against the EEC going further than merely making recommendations on the registration of all relevant research. However, following a colloquy held in May (see *Nature* 21 May, p.181) the Economic and Social Committee (ESC) is strongly advocating that the European Community adopt a legally binding text enforcing tight safety controls.

Both opinions will be taken into account by the ultimate decision-makers in the Committee of the Permanent Representatives to the EEC (Coreper) whose experts have been awaiting the views of the two consultative bodies.

The issue has been subject to an unusual amount of debate. In 1979 the European Commission itself proposed a legally binding directive along the lines demanded by ESC. This was then withdrawn and replaced by a set of recommendations to take account of evidence and scientific opinion which increasingly suggests that the dangers from bioengineering are less than were at first feared.

ESC thinks that the recommendations are too weak and that the reasons put forward to justify the original draft directive still hold good. It still considers that in the long term the unforeseeable and potentially serious consequences of recombinant DNA work require a "better safe than sorry approach", especially when pathogens are used as vectors or hosts. Also, the EEC has a responsibility to ensure that competition for commercially applicable research is not distorted by different rules on what can be done, and at what speed, in each member state.

The colloquy held by ESC to debate these points failed to budge the committee from its opinion, although many of the speakers there affirmed that the risks associated with genetic manipulation are small or negligible. The committee, however, remains convinced that transferring the techniques from laboratory to factory will not mean lower safety standards. Official guidelines would, therefore, be better than a system of self-regulation.

The report produced by Italian Euro-MP Domenico Ceravolo for the parliament echoes many of these concerns, but his resolution in favour of a directive was overturned in the committee vote by a majority of only one. In his report, Ceravolo attacks the European Commission's view by saying that even if a risk is only based on a hypothetical chain of events, this is no justification for thinking it any less valid or significant. And he argues further that the conjectural risks cannot be dismissed because no suitable criteria are available for assessing them.

Whether his arguments will win the day in the parliament's plenary session remains to be seen. A full vote was postponed at the last minute on 18 December but the vote is expected to go against Ceravolo. The liberals and conservatives, who form the majority in the house, support the Commission and feel that too much legislation will slow down the growth of Europe's biotechnology industry. The socialists and communists disagree and take their cue from the Italian left wing which sees EEC legislation as the best way of bringing Italian research under control.

Jasper Becker

Electronic publishing Journal plugs in

The British experiment to explore the feasibility of electronic scientific communication is well under way. The experimental electronic journal of the Universities of Loughborough and Birmingham, *Computer-Human Factors*, has received 16 papers in its first year — more than an earlier experiment in the United States received in its three-year life. Last week, the British Library, which is backing the experiment with £256,000, organized a demonstration for publishers,



journalists, editors and librarians.

The experiment is designed chiefly to identify the problems encountered by authors, editors, referees and readers in conducting editorial business on computers. Consideration will also be given to the possibility that electronic journals could fulfil the role of conventional journals. The experiment is also being used to investigate the role of computer networks for other less formal types of communication, such as newsletters, requests for comments on papers before submission to the journal, general communication between groups working on similar problems, the collaborative writing of papers and simply sending messages.

Members of the team working on the project say they are satisfied with the first year's results, ascribing their electronic journal's success in attracting papers to flexibility. In its purest form, an electronic journal would eliminate all paper; writing and editing would be done by means of VDUs (visual display units) and all transactions carried out over telecommunications links. Readers would also have access to the journal on their VDUs from a central computer memory.

Users of the journal preferring to see results on paper can get hard copy from printers at their terminals, and authors are also given flexibility by being allowed to submit papers either on-line or in the conventional way by posting typescripts to the editor. The editor can in-put perfect typescripts by optical character recognition but has to type in untidy ones on a word-processor. Of the 16 papers submitted so far, two have been on-line, and the rest came as typescript too untidy for optical character recognition.

The project, under editor Professor B. Shackel and his assistant Dr David Pullinger, is based at the University of Technology, Loughborough, and the central computer facility is provided by the University of Birmingham under the direction of Professor P. Jarratt. The 50 participants in the project, from universities throughout Britain, make up the journal's contributors and its only readers. Contributors are allowed to submit papers to conventional journals three months after submission to the electronic journal.

Although that option undermines the value of the electronic journal, its absence in the earlier United States experiment is thought to have dissuaded many potential contributors.

With two more years to run, the project is still at an early stage and the team is reluctant to draw many conclusions. Questions to be addressed, however, include the extent to which users can manage without paper, whether electronic journals could publish faster than conventional journals, the suitability of publishing papers and letters as soon as they are accepted rather than in batches as "issues" and alternative methods of refereeing.

Cost comparisons between electronic and conventional journals will be particularly difficult to assess. Capital cost could be minimized by using equipment initially acquired for other purposes, but running costs — chiefly the cost of using the telephone — will fall not only on the "publisher" but also heavily on users. One particular headache is how to compare the cost of reading time for conventional and electronic journals.

Even if this latest experiment demonstrates that electronic journals are feasible, the day when they become a practical reality in major subject areas is a long way off. The electronic journal, if it arrives, is likely to creep in gradually. Conventional journals, for example, may introduce new technology giving authors and readers the option of on-line access. But the problems of going entirely electronic are too formidable to be contemplated seriously for a few years yet.

Judy Redfearn

Creation science trial Verdict awaited

Washington

It may be another week before the verdict is known on the creationist trial which ended in Little Rock, Arkansas, last Thursday. Initially, Judge William Overton had promised an immediate verdict on whether a new state law requiring equal time for the teaching of evolution and "creation science" in state schools was unconstitutional.

At the end of the two-week trial, however, the judge announced that the amount of evidence presented was so large that his verdict would be delayed, although he has promised to deliver it by 31 December.

Despite the delay, the American Civil Liberties Union (ACLU), which brought the case on behalf of several local religious groups, biology teachers and school children, is confident that it has won. "It was no contest," Mr Bruce Ennis, the chief ACLU attorney, said after the trial had ended. "The state did what it could do. It was inadequate not because it did not do its job, but because creation science is a religion."

Supporters of creation science also

seemed to be accepting their defeat. But in this case the blame was being placed on the performance of state attorney general Steve Clark in defending the creation science law. The creationists promise a tougher fight in the next court case, which is likely to be a similar challenge against a creation science law passed in the state of Louisiana.

Although Judge Overton has yet to declare his verdict, he did say that it would be limited to the question of whether the creation science version of the origins of the world was religion, despite any explicit religious or biblical references in its description in the Arkansas law.

He added that he would not undertake to decide the validity of the biblical version of creation nor the theory of evolution. ACLU has asked the judge to determine various "findings of fact" — such as the definition of a scientific theory being based on natural laws and being "explanatory, testable and tentative" — which it hopes can be used in future court battles.

The second week of the trial was taken up largely by various witnesses called by the state to present a case in favour of creation science and the Arkansas bill, virtually identical copies of which are now pending before almost 20 other state legislatures.

Cross-examination by ACLU attorneys provided some colourful testimony. One supporter of creation science, having described how a creator could still be a scientific concept, perhaps comparable to Aristotle's "first cause", went on to describe his belief in exorcism and unidentified flying objects, claiming the latter to be attacks by Satan on God's world.

The star witness for the defence was Professor N.C. Wickramasinghe, head of the department of mathematics and astronomy at the University of Wales in Cardiff. Professor Wickramasinghe told the court that the odds against life originating by chance anywhere in the Universe were so high as to be virtually impossible. "One is driven almost inescapably to accept the possibility that life results from deliberate creation", he said.

He claimed that his own theories about the possible existence of microorganisms on comets bringing life to Earth had been rejected by other scientists largely because of their "indoctrination in Darwinism".

But if such statements were music to the ears of the creationists, there was less consolation when Professor Wickramasinghe was asked to comment on the creation science law, when he claimed that most of it was "claptrap", and that "certain parts of the law are demonstrably wrong".

One of the scientific witnesses who had been expected to appear for the defence unexpectedly left town shortly before he was due to testify. Another scientific witness whose appearance was cancelled by the state was Henry D. Voss, an electrical engineer who has published papers on space physics.

David Dickson

CORRESPONDENCE

Voices from a wilderness

SIR — We appeal for help to the editorial staff of *Nature*, to our colleague scientists and to the World Federation of Scientific Workers.

We are research workers of Jewish origin. More than two years ago, we came to the decision to leave the Soviet Union and accordingly applied for permission for our families to leave the country for Israel. Soviet law and present practices gave us reason to hope for a quick departure from the country since none of us has ever dealt with state or military secrets or with any questions connected with the security of the Soviet Union. Yet for more than two years we have been denied permission to leave, without reference to the Soviet legal code and without legal or lawful justification.

It seems that considerations in big politics do not take human lives into account.

We doubt whether our colleagues in the West realize what happens if a scientist in the Soviet Union declares his decision to leave the country. Almost all of us have lost our jobs and we are deprived of any possibility of continuing with our scientific research. The doors of scientific institutions, laboratories, seminars, conferences and symposia, editorial offices and publishing houses are shut in our faces. We are being deprived of our scientific degrees.

We feel that the danger of our creative death is becoming more and more real. Any scientist will understand what a two or three year break in scientific work means; and nobody can tell us how long our deprivation will last. Nobody is interested in our qualifications; nobody needs our work here.

Having lost our jobs, we find ourselves in a hard and humiliating financial position. We are anxious about the fate of our families; many of us have small children. We have in fact been thrown out of society, but at the same time we are not allowed to leave the Soviet Union.

All this has naturally had a harmful effect on our health — nervous depressions, hypertonic crises and other diseases have become our permanent companions. Our children are also affected.

We are in a situation where we can count only on the support and solidarity of our colleagues abroad, on their help and sympathy. Our voice in the Soviet Union is really a voice in the wilderness, our fate is of no interest to anybody and nobody answers

our complaints or petitions.

We understand that you all face important problems of your own, but hope that the fate of individual scientists — your colleagues — is also important and not a matter of indifference to you. For the world of science is common for everyone; it has common ethics and morals.

Indeed, our fate is closely connected with the attainment of lawfulness, the observance of the norms and rules of international pacts and human rights declarations, with the fulfilment of the Helsinki agreements and with adherence to humanitarian principles — with everything that makes possible mutual trust and mutual understanding between nations.

We are sure that struggle for the attainment of these principles is part and parcel of the struggle for peace and detente.

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Literary anarchy

SIR — The problems raised in recent letters (7 May, p.7, 28 May, p.278) about literature search and by Dana L. Roth (8 October, p.422) about recruiting librarians with subject expertise may be solved if and when today's librarians turn towards Anarchism as in the following: "How to be an Anarchistic but Indispensable Librarian" —

- (1) Make your own rules so that you can change and interpret them according to circumstances.
- (2) Always act contrary to the rules
- (3) Use flexible opening hours; go out as often as possible so that your readers are happy if and when they see you.
- (4) Differentiate your catalogue cards.
- (5) *a.* Articles in authors' names may or may not be written after the names; sometimes split names are typed as whole ones.
b. Use alternately full and abbreviated journal titles; use different abbreviations for the same title.
c. Keywords are typed above the author's name or underlined; singular, plural, adjective and complex word forms are to be used.
d. Never use see- or see also-references.
e. Never correct errors.
- (6) Foreign characters are alternately transliterated or not.
- (6) 1. Greek alphabet: transliterate or write in full ($\gamma = g$ or γ).
- (6) 2. Figures are alphabeticized before or after plain characters but they may also be written in full in different languages.
- (6) III. Chemical substances will be found alternately under the common name, the scientific name, the product formula or the gross formula.
- (7) Print a catalogue of current journal titles but do not include the years or volume numbers your collections start with.
- (9) Move your books and journals regularly.
- (X) Take holidays without warning anybody; never tell the date of your return.
- (11) Never compose statistics with exact numbers but make rough estimates.
- (12) Avoid management techniques (whatever they may be).
- (13) Help your readers as well as possible; talk at least half an hour with every new customer and at least one hour a week with regular visitors.
- (14) Be fraternal: send now and then somebody on to other libraries
- (eight) Never write anything down but don't try to remember everything either.
- (Four) Make sufficient catalogues: monographs and reprints each have separated authors, journals and serials, systematic, and keywords catalogues; put all these catalogues in one piece of furniture.

If you do this and a lot of other things, you deserve the title "Anarchistic Librarian"; but your customers will be satisfied and you have made yourself indispensable.

G. MERTENS

(Anarchist) Librarian

Katholieke Universiteit, Leuven, Belgium

SERC's plans

SIR — In your excellent leading article of 26 November (p.295) you state that the Science and Engineering Research Council "has just put off a plan to break new ground in the fashionable field of molecular electronics". It is certainly true that we are unable to fund in an adequate fashion programmes that certainly deserve support, but in the particular case of molecular electronics we are currently planning to increase our support of research in this potentially exciting area. Inevitably this means we have to cut back on other deserving areas.

B.W. OAKLEY

Science and Engineering Research Council, Swindon, UK

An acid test

SIR — The objection of Darnbrough *et al.* (*Nature* 26 November, p.294) to data in our *BioSystems* paper on the origin of life is based, according to correspondence, on experiments with ATP at pH 11, where ATP is unstable. On page 162 of the article, which they cite inaccurately, experiments at pH 7.2 are described. This technical detail (that is, experiments at pH 7.2) is critical in judging their statements about plausibility. For readers interested in confirming this, the correct reference is Fox and Nakashima, *BioSystems* 12, 155-166; 1980.

SIDNEY W. FOX

University of Miami, Florida, USA

NEWS AND VIEWS

Lymphokines on the move

from Verner Paetkau

LEUKOCYTES which are stimulated with mitogens or strong antigens have long been known to secrete mediators with profound effects on other leukocytes. Non-immunoglobulin protein products with effector properties in the immune system have been designated 'lymphokines' and have a wide spectrum of activities. It is perhaps not surprising that, until recently, only a few immunologists were interested in these mixtures of soluble mediators — they were apparently antigen-nonspecific and poorly defined. How things can change! Today, the lymphokines and related soluble mediators command the serious attention of both theoretical and practical immunologists. Several lymphokines and their relevant cells have been characterized, and a picture is emerging of discrete molecular entities acting in defined (or definable) ways. In addition, the factor identified as interleukin-2 (IL2), which stimulates proliferation of T lymphocytes, is used to generate clonal sources of T lymphocytes with specific cytotoxic or helper activities. Today, the interleukins are a growth industry.

The present phase of work began in the mid 1970s and results from the convergence of several lines of enquiry. The sources of factors were normal leukocytes stimulated with mitogenic plant lectins such as concanavalin A (Con A) or phytohaemagglutinin. Not unexpectedly, the conditioned culture media used in early experiments contained a variety of activities, and different laboratories concentrated on different lymphokine assays. In one case, a soluble mediator greatly enhanced normal mitogenic responses of thymic lymphocytes to certain plant lectins¹. In another, lymphokine preparations generated from mitogen-stimulated leukocytes were found to induce the continuous proliferation of functional T lymphocytes^{2,3}. Similar preparations could also replace T lymphocytes in antibody-forming responses of B lymphocytes⁴, an activity referred to as 'T cell replacing factor' (TRF).

By 1978, it was evident that at least two molecular entities could be defined. One, a product of macrophages or monocytes, had been termed 'lymphocyte activating

factor'. The factor responsible for this activity has been renamed interleukin-1, and was recently purified to homogeneity⁵. The other, with effects on thymic lymphocytes, cytotoxic lymphocytes and T-depleted B lymphocytes, bore a number of designations, depending on the assay and laboratory. A single factor appeared to be responsible for this group of activities, there being no resolution by gel filtration, ion exchange chromatography, or isoelectric focusing. It was designated interleukin-2 (for T cell growth factor, thymocyte stimulating factor and co-stimulator). This was more than an exercise in semantics; it helped to focus the work and to give it some testable tenets. Because both biochemical and biological criteria for distinguishing IL2 and IL1 existed, it was possible to look for other activities in crude culture supernatants. Recent progress has been both stimulating and satisfying.

A soluble mediator which induces an enzyme activity (20- α corticosteroid dehydrogenase) associated with mature helper T lymphocytes has now been characterized by Hapel and co-workers⁶, and designated IL3. Conveniently, IL3 and IL2, which are both present in Con A-generated supernatants of normal spleen cell cultures, are separable by ion exchange chromatography. IL3, which is demonstrably free of IL2, interferon and TRF, stimulates the clonal outgrowth of T lymphocytes with surface antigens expected of T helper cells. Indeed, the clones eventually begin to generate their own IL3 and become able to grow independently of added factor. These putative T helper cells produce IL2 when stimulated with phorbol myristate acetate (PMA), an agent previously shown to elicit IL2 production from normal splenic T (helper) lymphocytes⁷. PMA apparently overrides the normal requirement for IL1 and antigen/mitogen for IL2 production⁸. Given the availability of biochemical separation methods and clonal cell lines for assay, one can soon expect a further

understanding of T cell signal networks, in which the roles of interferon, colony stimulating factor, IL1, I-A recognition, IL2, IL3, TRF and antigen will be identified more precisely.

The question of whether IL2 acts directly on B cells when it restores T cell function in antibody responses has not been answered definitively. The difficulties can be illustrated by recent work in which spleen cells of athymic mice were stimulated to grow by IL2 preparations, and in the process generated T lymphocytes (Lipsick and Kaplan, ref.9). This observation is consistent with previous results showing a recovery of T cell function *in vitro* and *in vivo* with IL2. The recovered T cell function may in turn be responsible for the restoration of T-dependent B cell function.

Does this report show, in contrast to what had been thought, that IL2 is directly mitogenic for T cells or their precursors in the absence of a second signal (antigen or mitogen)? The difference from earlier reports may be less significant than it seems. It is known, for example, that IL2 has no direct mitogenic effect at low cell density. In the Lipsick and Kaplan work, spleen cells were cultured at high density (5 million per ml) in serum-free medium; serum both enhanced background mitogenic activity and reduced that stimulated by IL2. A significant question remains — is the activity in fact due to IL2? Or, in light of the results of Hapel *et al.*⁶, is IL3 at work? Although most of the experiments were performed with relatively crude IL2, which may well contain IL3, the same results were apparently obtained with preparations which should be free of IL3. Further direct evidence on this point will be welcome.

The question of direct effects of IL2 on B cells may be partly resolved by the finding that IL2 preparations contain a discrete factor which stimulates B cell proliferation¹⁰. Similar results are forthcoming from laboratories at the NIH (Bethesda) (M. Howard *et al.*, personal communication). The B cell growth factor has been separated from IL2 (ref. 11). In the model studied, the NIH workers feel that IL2 is synergistic with B cell growth factor.

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Finally, what of TRF⁴? After many years of difficulty, created in part by the ability of factors like IL2 and the B cell growth factor to replace T cells indirectly, assays which clearly distinguish between IL2 and TRF are now in hand. And the take-home message is that, indeed, TRF exists¹². It acts at the time when B cells, having undergone clonal expansion following antigenic stimulation, are to begin secreting high levels of immunoglobulin. Then, as proposed years ago by Dutton and colleagues, TRF can replace T cells in switching on a high level of immunoglobulin synthesis. Using spleen cells thoroughly depleted of T cells (this requires treatment of the donor animal with anti-thymocyte serum, in addition to the more common treatment with anti-Thy-1 antibody *in vitro*), Swain and co-workers have demonstrated synergy between IL2 and TRF in generating plaque-forming B cells. The role of IL2, although not yet delineated in this system, may be related to its ability to induce T cell

functions early (proliferative phase). The relationship to the newly described B cell growth factor awaits further experiments. In both cases, a clearer understanding of the roles of several lymphokines now exists, and perhaps more important, the tools and approaches necessary for more sophisticated analysis are evident.

An indication of future directions is

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12. Swain, S.L., Denner, G., Warner, J.F. & Dutton, R.W. *Proc. natn. Acad. Sci. U.S.A.* **78**, 2517 (1981).
13. Farrar, J.J. *et al. J. Immun.* **125**, 2555 (1980).
14. Bleackley, R.C. *et al. J. Immun.* (in the press).

given by a current report describing a start on molecular biological studies of IL2. Given several T cell tumour lines which can be induced to generate high levels of IL2 (several thousand times the amounts produced by normal lymphocytes), it has been possible to follow the lead of workers in the interferon field, by looking for mRNA coding for the lymphokine, and then attempting to clone its complementary DNA. The EL4 variant cell line originally described by Farrar and colleagues at NIH¹³ does indeed contain demonstrable IL2 mRNA. The poly (A)⁺, 11–12S RNA derived from PMA-stimulated cells of this line, injected into *Xenopus laevis* oocytes, directs the synthesis of biologically active IL2¹⁴. Similar results have been obtained by Gillis and colleagues in Seattle. The next phase of work clearly will involve molecular biological approaches. With venture capital panting for release, one may also expect that the term 'growth industry' could soon apply to lymphokinology in more than one way. □

A new view of supernova remnants in the Magellanic Clouds

from David Clark

FEW areas of astrophysics have not seen major changes following the highly successful Einstein X-ray observatory mission. The impact on the study of supernova remnants (SNRs) has been particularly impressive. When a massive star dies as a supernova, a shock wave is driven outwards from the site of the explosion, sweeping-up and heating interstellar gas to the millions of degrees required for thermal X-ray emission — so forming an extended X-ray remnant with characteristic peripheral brightening. Gas cooling behind the shock may be seen to radiate optically, and SNRs may also be detected at radio wavelengths. The radio emission is non-thermal, produced by fast electrons trapped in the interstellar magnetic field compressed by the expanding shock wave.

Because X-ray astronomy is a comparatively young science, pioneering surveys of SNRs in our own Milky Way, and its closest galactic neighbours, the Magellanic Clouds, were carried out in the radio and optical. Within the Galaxy more than 120 extended non-thermal radio objects were identified as SNRs. Detectable optical nebulosity was found only in the 30 closest of these because of the obscuring effect of dust permeating the plane of the Galaxy. A common characteristic of the optical remnants was recognized — the intensity of the red line emission associated with singly ionized sulphur with respect to the intensity of the red hydrogen Balmer line was very much greater in the shock-ionized plasma of SNRs than in photo-ionized nebulae. The combination of non-thermal radio emission and strong sulphur emission was taken to be a unique signature of SNRs,

and was used to detect remnants in the Magellanic Clouds and other nearby galaxies.

The pioneering survey of SNRs in the Magellanic Clouds by Mathewson and Clarke in Australia in 1973 used the radio/optical technique described above. The survey identified 14 remnants in the Large Magellanic Cloud (LMC) and 3 in the Small Magellanic Cloud (SMC), and was assumed to be almost complete (although a few other ring-like nebulosities seen in a deep optical survey of the Clouds by Davies and co-workers in 1976 were proposed as possible remnants). That assumption has been shattered by the X-ray survey of the LMC completed by Einstein and recently reported by Long, Helfand and Grabelsky (*Astrophys J.* **248**, 925; 1981). Einstein detected X-ray emission from 13 of the known 14 LMC remnants, plus 12 other objects which are certain SNRs (5 of which were also in the Davies *et al.* list), plus 25 further objects which are possible SNRs on the basis of existing X-ray data.

The implications of these new discoveries are very significant. They seriously question the sole use in extragalactic SNR surveys of the traditional radio/optical SNR detection technique as it revealed perhaps just one-quarter of the LMC population of remnants. Optical nebulosity associated with many of the new remnants showed sometimes no sulphur emission — sometimes no hydrogen

emission. The new detections allow the supernova rate in the LMC to be lowered to perhaps one per century, contrasting with earlier estimates of one per 400–900 years. But most significant, the major population of LMC SNRs now identified in X rays and optically are found to be radio 'sub-luminous'; this result must call into question many of the studies of remnant evolution, distribution, ages, birth rates, distances and so on in our own Galaxy since such studies are based almost entirely on radio data. The new uniform LMC sample of SNRs should prove invaluable in future studies of remnant evolution at all wavelengths.

Observations of the six brightest LMC remnants using the solid-state spectrometer on Einstein are to be published shortly. These too show unexpected results. Four of the six display characteristic thermal spectra expected for shock-heated interstellar material; but the other two show non-thermal spectra suggestive of continued energy input. This is the case for the well known SNR the Crab Nebula, which in addition to emitting non-thermal X rays is brightest at its centre rather than peripherally, the emission being driven by an active pulsar. One of the LMC remnants, going by the rather unspectacular name of N157B, also shows unusual central brightening (as Australian radioastronomers showed several years ago) and mimics the Crab Nebula in other ways, including the non-thermal X-ray emission. It is the first Crab-like remnant detected in another galaxy, and must represent a prime site for the detection of the first extragalactic pulsar. □

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Are stress fibres contractile?

from Keith Burridge

INTEREST in stress fibres arises in part because their organization resembles the structure of striated muscle myofibrils. Not only do they contain many of the same proteins (for example, actin, myosin, α -actinin and tropomyosin) but many of these are arranged in an alternating periodic pattern similar to that seen in sarcomeres. The similarity to muscle structure has suggested that they may have a similar function and be involved in non-muscle cell movement.

Here, however, is a paradox. Stress fibres are not required for cell movement or migration, and rapidly moving cells such as macrophages or amoebae lack them. Couchman and Rees¹ have shown that fibroblasts migrating from primary tissue explants generally lack stress fibres in the first few days when they are migrating rapidly. With time the migration of the cells slows down and stress fibres become more prominent. The correlation of lack of movement with the presence of stress fibres has been best documented by Herman, Crisna and Pollard². Fibroblasts migrating randomly in culture were filmed, then fixed and prepared for immunofluorescence with antibodies against actin, and examined for the presence or absence of stress fibres. Cells or regions of cells moving most actively appeared to contain no stress fibres while regions of cells with prominent stress fibres moved very little.

Because of the similarity of stress fibres to myofibrils, several groups have looked for contractility. Isenberg and co-workers³ demonstrated that stress fibres which have been microdissected from living cells with lasers will contract in the presence of Mg^{2+} -ATP. An elegant approach was used by Kreis and Birchmeier⁴ who microinjected fluorescently labelled α -actinin into living cells so that it would be incorporated into the stress fibres. Contraction was then induced in permeabilized cell models with Mg^{2+} -ATP. During such contractions, shortening of the non-fluorescent space between the fluorescent regions of α -actinin was observed, consistent with a sliding filament mechanism like that found in muscle. Contraction was not, however, observed in live cells as they first had to be permeabilized. Clearly these structures are potentially contractile, but what is their function in intact cells?

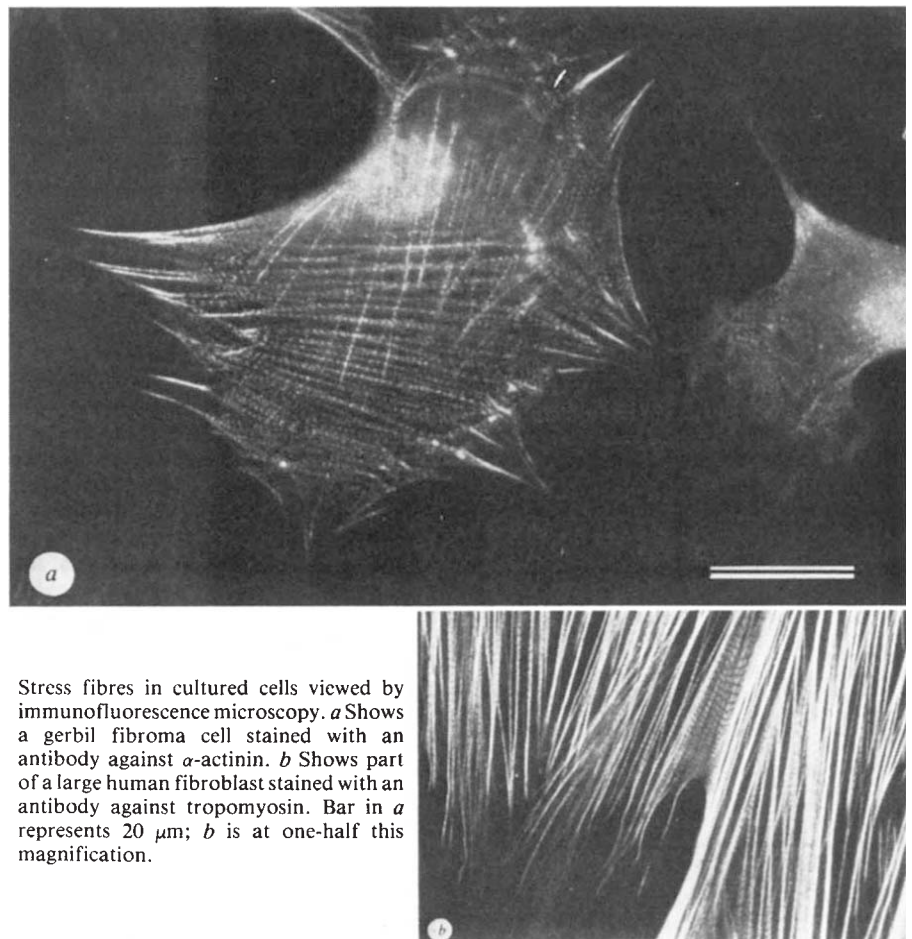
It would be helpful to know under what physiological circumstances stress fibres are found in the body but, unfortunately, rather little information is available. Stress fibres have been described in aortic

endothelial cells of hypertensive animals⁵ and also in the granulation tissue of wounds⁶ but fibroblasts in many normal tissues do not contain prominent stress fibres. Growth of fibroblasts in culture seems to promote the development of stress fibres, perhaps reflecting the unphysiological conditions of the culture environment. Unlike conditions *in vivo*, cultured cells grow on a rigid substrate to which normal fibroblasts adhere strongly. Some years ago, Willingham and his co-workers⁷ showed that when the substrate was weakly adhesive for the cells, they did not spread and did not form stress fibres. When the same cells were induced to adhere tightly, they flattened on the substrate and prominent stress fibres appeared. Tight adhesion to the underlying substrate appeared to be the key to their appearance. Couchman and Rees¹ reached a similar conclusion in their study of fibroblasts migrating from explants.

How might tight adhesion to a substrate induce the formation of stress fibres? One possibility is that the tight adhesion itself restricts or sterically inhibits the local movements of surface membranes. In turn, this might permit an ordering of the adjacent cytoskeletal elements into stress fibres — one that would not occur in a

region of cytoplasm that was in a more dynamic state, such as that underlying a ruffling membrane. An alternative explanation I find preferable is that stress fibres arise within a cell because it attempts to pull against a point of tight adhesion to the substrate. Since the substrate is a plastic culture dish, tension will develop. This will result in the alignment of individual microfilaments and possibly also in the recruitment of additional filaments to the bundle that develops. Thus the more strongly a cell adheres to a substrate the greater is the tension and the larger the resulting stress fibre. Several lines of evidence are consistent with this view.

Wohlfarth-Botterman and Fleischer^{8,9} showed that tension will induce large bundles of microfilaments in the protoplasmic strands of *Physarum* when these are made to contract under isometric conditions. The microfilament bundles, reminiscent of the stress fibres of fibroblasts, are not induced, however, during isotonic conditions. *Physarum* is phylogenetically far from cultured fibroblasts, but it may reveal a principle of non-muscle actomyosin systems. When stress fibre contractility in fibroblasts has been studied, isotonic contraction (that is, shortening) has been looked for, but the failure to observe this in live cells is hardly surprising if tight adhesions to the substrate make shortening impossible. Strong adhesions would impose isometric conditions on a contraction if the



Stress fibres in cultured cells viewed by immunofluorescence microscopy. *a* Shows a gerbil fibroma cell stained with an antibody against α -actinin. *b* Shows part of a large human fibroblast stained with an antibody against tropomyosin. Bar in *a* represents 20 μ m; *b* is at one-half this magnification.

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adhesions do not break. Only when these adhesions have been weakened, as might be expected in cell model studies^{3,4}, would shortening of stress fibres be observed.

That tension on the substratum is generated has been shown by Harris and co-workers^{10,11}, who introduced flexible silicone rubber substrates to study cell movement. Highly motile cells, such as leukocytes, are unable to deform the flexible substrate during their movements, but more sedentary cells such as fibroblasts cause extensive wrinkling of the rubber, indicative of considerable tension. It seems most likely that stress fibres are the cellular structures responsible for generating this tension. Cells without stress fibres, even though they may move rapidly on the flexible substrate, appear unable to generate sufficient tension to wrinkle it. On the other hand, those cells that induce wrinkles beneath them tend to show large microfilament bundles often running at right angles to the wrinkles (unpublished observations). If the cells are rounded up with trypsin, the wrinkling (that is, the tension) is released¹¹ and others have shown that these conditions also result in the rapid disassembly of stress fibres (see, for example, ref. 4). To generate the wrinkle some shortening of the stress fibres must occur; but even here a considerable element of the contraction must be isometric, since once formed the wrinkles are held for long periods of time, although they will snap back if the cell's adhesion to the rubber is released, indicating a sustained tension both in the rubber and the cellular elements causing it to wrinkle.

In conclusion, it would seem that stress fibres have little to do with cell migration. Several lines of evidence suggest that these structures are contractile but that in cultured cells this contraction is isometric rather than isotonic. It seems likely that the tension generated at points of tight adhesion to the substrate contributes to the formation of these bundles of actin microfilaments. In future it will be important to investigate further the conditions in which stress fibres appear in the body, and it will be particularly relevant to examine locations where tight adhesion and isometric tension might be expected to occur. □

Order or disorder in the structure of glass?

from S.R. Elliott

THE question posed in the title has provided a recurrent theme in the study of the structure of non-crystalline solids since the seminal work of Zachariasen in 1932. The absence of long-range order (periodicity) is a prerequisite for an amorphous material. The point at issue, therefore, is what degree of short- or medium-range order is present, if at all, in a glass? Is the structure of non-crystalline materials completely random and chaotic, or are there, as Zachariasen supposed for covalent materials, well defined structural units (for example, SiO_4 tetrahedra in SiO_2 glass) which are themselves randomly connected together?

Much evidence from diffraction and other experiments shows that covalently bonded amorphous solids do possess a strong degree of local order (that is, structural units), but controversy still exists over the degree of medium-range order — whether the units are completely randomly connected together, or whether there exist preferred orientations, just as in the crystalline forms.

It is generally accepted, following Zachariasen, that the structure of directionally bonded (that is, covalent) amorphous solids can be well represented by a 'continuous random network' (CRN), in which say for SiO_2 , SiO_4 tetrahedra are connected together at the apices through the oxygen atoms, such that every Si is surrounded by four O atoms, and every O is surrounded by two Si atoms, although the bond-angle subtended at the O atom may subtend a wide range of values. The SiO_4 tetrahedra is relatively well defined but there exists some uncertainty in the Si bond-angles, and the relative orientation of units is not at all well defined. A further complication arises for multicomponent systems since a single diffraction experiment only gives an average picture of the structure, being a sum of say, the individual Si-Si, Si-O, O-O pair correlation functions, so that only a limited amount of unambiguous structural information can be obtained.

For these reasons, a recent paper by Daniel and Leadbetter¹ makes an interesting contribution. They studied an amorphous elemental system, arsenic, by means of X-ray diffraction. Amorphous arsenic (a-As) has been much studied in the past (see ref. 2 for a review), and is of interest because of its position in the Periodic Table midway between a-Si (used for solar cells) and a-Se (the active component in the Xerox process). However, in contrast to previous studies, Daniel and Leadbetter studied the molecular form of a-As. It is well known that As vapour consists of tetrahedral As_4 molecules, and if this vapour is condensed on a substrate held at 30K, an amorphous

form is produced consisting of a packing of As_4 molecules, bonded together by van der Waals' forces. Thus, in this case, the local order is extremely well defined (that is, the As_4 tetrahedron), and in principle the medium-range order of the amorphous solid can be explored directly, since the local structure is known accurately. A model whereby all the As_4 tetrahedra are randomly oriented does not give peaks in the angular dependence of the scattering intensity in the same positions as those observed experimentally, implying there is a considerable degree of correlation between molecules in the way they pack. Instead, it appears that the molecules of As_4 prefer to pack in a slightly distorted, staggered face-to-face configuration. Similar substantial correlations are found in other (liquid) molecular systems, such as P and CCl_4 .

Another interesting point is the observation that molecular amorphous arsenic is structurally unstable, even at temperatures as low as 77K. The highly strained As_4 molecules, with bond-angles of 60° , irreversibly polymerize to form a cross-linked network, which has an X-ray diffraction pattern very similar to that of bulk a-As, which has a bond-angle of 98° and whose structure can be well simulated by a CRN². This amorphous-amorphous transition is interesting in that it involves a distinct change in medium-range order: peaks in the real-space correlation function separate much further in the molecular form than in the polymerized material.

This work has considerable bearing on another commonly studied amorphous system, the As_2S_3 system. This, unlike As itself, readily forms a glass by quenching of the melt, but can also be formed into amorphous thin films by vacuum evaporation. However, the stable vapour species is the almost spherical As_4S_6 molecule, not the As_2S_3 molecule which would preserve stoichiometry, and indeed Daniel and Leadbetter in an earlier paper³ studied the structure of evaporated amorphous thin films of ' As_2S_3 ' and concluded that the structure is composed of a packing of As_4S_6 molecules. Just as in the case of elemental As films, the As_4S_6 molecules appear to pack in a highly correlated manner, but detailed interpretation is made difficult because of the two atomic species present and also because of the spherical nature of the molecule concerned.

Evaporated thin films of As_2S_3 (or other alloys containing S or Se) are interesting because they exhibit both thermally and photo-induced structural changes. Like molecular As, the As-deposited films polymerize irreversibly on annealing, the

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material becoming more chemically ordered in the process, as shown by X-ray diffraction³ and EXAFS studies⁴. These materials are semiconductors, and irradiation by band-gap light also serves to induce polymerization, the so-called 'irreversible photostructural effect'. More mysterious, however, are the reversible structural changes induced by band-gap radiation on well annealed films and melt-quenched glasses of the same materials. These structural changes, on the scale of about 6 Å appear to be smaller than those encountered in the irreversible effect and are accompanied by various optical

changes⁵; they can be annealed away by heating the sample to the glass transition (softening) temperature, whereupon re-excitation induces the same effect. The nature of these structural changes remains completely unknown. Perhaps when we understand the medium-range order present in glasses we might begin to understand this effect too! □

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Calcium and calmodulin in Kyoto

from Robert S. Adelstein

MANY diverse biological processes are regulated by the concentration of intracellular calcium, including cell motility and the synthesis and degradation of biologically important peptides and nucleotides. The effects of calcium are often mediated by the small (molecular weight 16,500), acidic protein calmodulin, which appears to be ubiquitous throughout much of the plant and animal kingdoms. Calmodulin contains four binding sites for calcium which become occupied at a calcium concentration of 10^{-6} M, a level corresponding with the onset of many calcium-mediated processes in cells. In marked contrast to the calcium-free molecules, the complex of calcium and calmodulin is capable of binding to and activating numerous proteins with different biological activities. The mechanism and consequences of the binding of calcium to calmodulin were discussed in a meeting* in Kyoto, Japan this past summer.

There was general agreement that positive cooperativity has a role in binding the second Ca^{2+} to calmodulin (K. Yagi, Hokkaido University; J. Haiech, Montpellier University). It was further proposed that the binding of all four Ca^{2+} to calmodulin is not random, but occurs in a specific sequence. Two enzymes that are activated by calmodulin, cyclic nucleotide phosphodiesterase and myosin kinase, appear to require the species with four Ca^{2+} bound.

A major theme recurring throughout the meeting was the interaction between two major intracellular regulators: Ca^{2+} , which is often coupled to calmodulin, and cyclic nucleotides, which are coupled to enzymes catalysing covalent phosphorylation. The kinetic analysis of the regulation of cyclic nucleotide phosphodiesterase by calcium and calmodulin was

presented by J. Wang (University of Manitoba) and his model used to explain the highly cooperative activation of phosphodiesterase by Ca^{2+} -calmodulin.

In the control of serotonin biosynthesis (Yamauchi and Fujisawa, Asahikawa Medical College), both tyrosine and tryptophan hydroxylases appear to be regulated by a Ca^{2+} -calmodulin-dependent phosphorylation which promotes the binding of a separate activator protein. This heat-labile activator has a molecular weight of 70,000, is composed of two subunits of 35,000 and binds to the phosphorylated species of hydroxylase only, bringing about a twofold increase in enzyme activity. Additional activation by cyclic AMP-dependent phosphorylation at other sites provides a separate mode for regulating these enzymes.

Y. Takai and Y. Nishizuka (Kobe University School of Medicine) described a calcium-dependent but calmodulin-independent membrane-bound kinase. This enzyme, which has now been purified from rat brain to near homogeneity, has a molecular weight of 77,000 and has been named C-kinase. It requires micromolar concentrations of Ca^{2+} and phospholipid for its activation, but contains no calmodulin, nor is it activated by exogenous calmodulin. Instead, a small quantity of unsaturated diglyceride increases the affinity of enzyme for Ca^{2+} as well as for phospholipid. The hydrolysis of phosphatidylinositol to phosphatidylserine and diacylglycerol, which can be induced by a number of different extracellular messengers, is directly coupled to its activation. The presence of relatively large amounts of protein kinase C activity in brain, platelets and other tissues and the interaction of this system with the cyclic GMP-regulatory system, suggests great promise for further studies.

Ca^{2+} - and cyclic nucleotide-mediated reactions appear directly coupled at the level of their receptor proteins (C. Klee,

NIH). In brain extracts, calmodulin interacts in a Ca^{2+} -dependent fashion with the regulatory subunit of cyclic AMP-dependent protein kinase.

One of the best examples of the coupling of the Ca^{2+} -calmodulin regulatory systems with cyclic AMP-mediated regulation is that of smooth muscle. Myosin kinase, the enzyme regulating phosphorylation of smooth muscle myosin, is a substrate for cyclic AMP-dependent protein kinase (R. Adelstein, NIH) and phosphorylation of myosin kinase decreases its activity by decreasing its ability to bind calmodulin.

The role of calmodulin in regulating muscle contractile proteins was discussed by several investigators. Calmodulin-dependent phosphorylation of the 20,000 molecular weight light chain present on both heads of a single smooth muscle myosin molecule appears to be required before the Mg^{2+} -ATPase activity of either head can be activated by actin (D. Hartshorne, University of Arizona). Using tracheal smooth muscle (J. Stull, University of Texas) an increase in tension was found to be paralleled by an increase in myosin phosphorylation; however, consistent with the results of Dillon *et al.* (*Science* **211**, 495; 1981), it was possible to maintain isometric tension despite a decrease in the state of myosin phosphorylation.

S. Kakiuchi (Osaka University Medical School) presented evidence that calmodulin binds to spectrin, a component of the erythrocyte membrane, and suggested that the interaction might be involved in maintaining the normal shape of the red cells. He also reported the presence in gizzard smooth muscle of a novel protein (molecular weight 150,000) that binds to calmodulin in the presence of 10^{-6} M Ca^{2+} but binds to actin at low ($<10^{-7}$ M) Ca^{2+} concentrations. The protein might inhibit the ability of actin to polymerize at low Ca^{2+} concentrations.

Pharmacological aspects of the calmodulin system opened the meeting. F. Vincenzi (University of Washington) reappraised the action of neuroleptic drugs, particularly phenothiazines on calmodulin function. Some presumed calmodulin antagonists as well as neutral and acidic lipids seem to act directly on activatable enzymes, possibly interacting with hydrophilic regions of calmodulin-binding sites.

H. Hidaka (Mie University Medical School) presented studies of naphthalene-sulphonamide derivatives (W-7 and W-5) as antagonists of calmodulin action. These amipophilic compounds appear to be more selective for calmodulin than phenothiazines with much less effect as general membrane perturbants. Treatment of cultured Chinese hamster ovary cells

*The symposium on Recent Advances in Ca^{2+} and Cell Function: Calmodulin and Intracellular Ca^{2+} -Receptors was held in Kyoto, Japan and organized by S. Kakiuchi (Osaka University Medical School) and H. Hidaka (Mie University School of Medicine).

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with W-7 at moderate concentrations was shown to prohibit cell growth, arresting the cells in late G₁ phase. Using ³H-labelled W-7, its distribution *in vivo* was shown to be similar to that of calmodulin under

conditions where growth arrest was obtained — suggesting that calmodulin may provide Ca²⁺-dependent regulation of functions required for the cells commitment to replication and division. □

Environmental nitrosamines and cancer

from Valda M. Craddock

It is now twenty-five years since dimethylnitrosamine was shown to cause liver cancer in rats in the historic experiments of Barnes and Magee. Tumours have now been shown to be induced by a wide variety of *N*-nitroso compounds in many species of animal and there is no reason to believe that man will prove to be an exception. There is also no doubt that man is exposed to these potent carcinogens. They frequently occur in industry and are widespread in the environment. Clearly, it is important to find out where the nitroso compounds come from, whether the levels found represent a hazard for man, what can be done about them and what the cellular and molecular mechanisms are by which they cause cancer. Progress in these areas was

discussed at a recent conference* in Japan.

At previous meetings, emphasis had been on methods for the detection and estimation of nitrosamines when present in complex biological material. In the past, several reports of nitrosamines in the environment have proved to be false alarms when more specific analysis showed the presence of related chemicals. The situation at present is that volatile nitrosamines can be analysed by gas chromatography/thermal energy analysis. With non-volatile nitrosamines, preliminary formation of volatile derivatives is necessary.

N-nitrosoamines are likely to be formed wherever amines, secondary or tertiary, encounter nitrite. *N*-nitrosamides result from similar reactions with amides. Both types of compound are potent carcinogens. Nitrosamines are relatively stable and require metabolic activation to form the ultimate carcinogen; nitrosamides on the other hand are unstable, and the site of

carcinogenicity is, in general, independent of metabolic action.

Much good work done in detecting environmental nitrosamines comes from the German Cancer Centre (Preussmann, Heidelberg). Nitrosamines in beer were first detected in Germany. They are formed by the reaction of amines in barley with nitrous fumes from the fuel used to heat barley during the malting process, and can be dramatically reduced by changing the source of the heat. The nature of the amines involved is being elucidated (Wainwright, Brewing Research Foundation, Nutfield). Nitrosamines have been detected in rubber, including the rubber used in the manufacture of babies' dummies (Spiegelhalter and Preussmann, German Cancer Centre, Heidelberg). The use of alternative retarders, or of 'safe' amines, should reduce this hazard. Relatively high concentrations of nitrosamines have been found also in cutting oils (Keefer, NIH), and it is again possible to suggest methods for their elimination. Cosmetics are known to contain nitrosamines, and here the situation is less clear. The fact that female facial skin is not a high cancer incidence area is not necessarily a reassurance; *N*-nitroso compounds can be absorbed through the skin and cause cancer elsewhere. On the whole, however, the meeting was

*Seventh International Meeting on *N*-Nitroso Compounds: Occurrence and Biological Effects, held in Tokyo, 28 September–1 October 1981, under the sponsorship of the International Agency for Research on Cancer, France, and the Japanese Cancer Association, Tokyo.

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100 years ago

THE VOYAGE OF THE "VEGA" AROUND ASIA AND EUROPE

By A.E. Nordenskjöld (Macmillan & Co., 1881)

THE voyage of the *Vega* will be in many respects one of the most memorable events in the history of navigation. For the first time a continent has been circumnavigated, so far as authentic record goes, and at last the North-East Passage has been won, after heroic efforts begun nearly three and a half centuries ago. But the voyage will be still more memorable by the two rich volumes in which it finds copious record, volumes which have scarcely a parallel in the whole literature of geographical exploration.

In one chapter, Baron Nordenskjöld has collected all the information attainable on Steller's sea-cow (*Rhytina Stelleri*), which on Steller's visit to Behring Island in 1741 was found pasturing in large herds on the abundant sea-weed on the shores of the island. Twenty-seven years after, not a specimen was to be found, and it was believed to be then

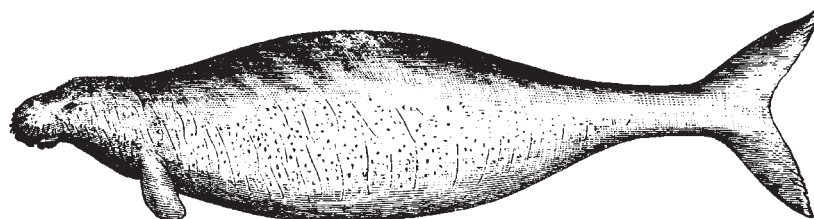


FIG. 10.—Reconstructed form of the sea-cow.

extinct. But Baron Nordenskjöld adduces evidence to prove that a specimen was seen twenty-seven years ago, though there can be little doubt that it has really gone the way of the mammoth. The Baron does not believe that its extinction is due to the destruction by hunters, but that it was a survival from a past age doomed to extinction, which overtook it when driven from its pastures on the shores of Behring Island.

"Steller's sea-cow (*Rhytina Stelleri*, Cuvier) in a way took the place of the cloven-footed animals among the marine mammalia. The sea-cow was of a dark-brown colour, sometimes varied with white spots or streaks. The thick leathery skin was covered with hair which grew together so as to form an exterior skin, which was full of vermin and resembled the bark of an old oak. The full grown animal was from twenty-eight to thirty-five English feet in length and weighed about sixty-seven cwt. The head was small in proportion to the large thick body, the neck short, the body diminishing rapidly behind. The short fore-leg

terminated abruptly without fingers or nails, but was overgrown with a number of short thickly placed brush-hairs; the hind-leg was replaced by a tail-fin resembling a whale's. The animal wanted teeth, but was instead provided with two masticating plates, one in the gum the other in the under jaw. The udders of the female, which abounded in milk, were placed between the fore-limbs. The flesh and milk resembled those of horned cattle, indeed in Steller's opinion surpassed them. The sea-cows were almost constantly employed in pasturing on the sea-weed which grew luxuriantly on the coast, moving the head and neck while so doing much in the same way as an ox. While they pastured they showed great voracity, and did not allow themselves to be disturbed in the least by the presence of man. One might even touch them without their being frightened or disturbed. They entertained great attachment to each other, and when one was harpooned the others made incredible attempts to rescue it."

From *Nature* 25, 22 & 29 December, 1881.

reassuring in that it showed competent groups to be tracking down environmental nitrosamines, and to be suggesting realistic methods for reducing their formation.

More difficult to elucidate is the formation of nitrosoamines *in vivo*. Amines are ubiquitous in biological material, and therefore occur in vegetable and animal food. Nitrate occurs in plant material and in water, and can be reduced to nitrite by bacteria in the gastrointestinal tract, especially in the mouth. Hence amines and nitrite are likely to react together in the stomach. The amount of nitrosamines formed depends on the nature of the stomach contents, and has been shown to be altered by gastro-duodenal diseases (Walters, Food Research Association, Leatherhead). As nitroso compounds are usually rapidly metabolized, and are formed in small amounts at any one time, their estimation is difficult. An ingenious method is being developed in which the naturally occurring amino acid proline is given to experimental animals or to human subjects. Nitrosation of this amine yields *N*-nitrosoproline, a nitrosamine which is apparently not carcinogenic and which is excreted in urine. Determination of urinary *N*-nitrosoproline could therefore give a measure of the formation of *N*-nitroso compounds in man (Ohshima and Bartsch, International Agency for Research on Cancer, Lyon). Where reduction of the nitrate content of food would lead to other problems, as in the case of cured meat, it is possible to add compounds, such as ascorbic acid, which, in certain conditions, reduce nitrosamine formation (Tannenbaum, MIT).

Many useful drugs are amines which can give rise to carcinogenic nitrosamines. Here the only solution at present seems to be a risk/benefit evaluation with each drug, possibly with each patient. To take cimetidine for dyspepsia, whether or not an ulcer is known to be the cause, or to take this drug indefinitely to prevent recurrence of an ulcer, requires justification.

The levels of nitrosamines to which man is exposed may be low at any one time, but the effect of one nitrosamine given at different times, and of different nitrosamines administered simultaneously, can be additive. Hence to ask each time nitrosamines are detected in a product, "is this level likely to constitute a hazard to man?", is meaningless. The nitrosamines consumed in Scotch whisky must be added to those in bacon, mushrooms, cigarette smoke, cosmetics, and to the nitrosamines formed *in vivo*.

While the entire population is probably exposed to nitrosamines in some form, everyone does not get cancer. The more that is understood about the means by which nitrosamines cause cancer, the more it is possible to detect high-risk individuals or groups, and to take protective measures. The best working hypothesis is that metabolism of nitrosamines leads to the formation of alkylating species which react

with DNA and cause mutation-like events which disrupt certain control mechanisms in the cell.

The first biochemical step in carcinogenesis, the metabolism of the aliphatic, heterocyclic, or aromatic nitrosamines, has been studied by chemical *in vivo* and *in vitro* methods (Okada, Tokyo Biochemical Research Institute; Preussmann). Oxidation occurs at various carbon atoms, but it was pointed out that the major metabolites may represent genuine detoxication reactions, while metabolites produced by minor pathways, and which would be more difficult to detect, may represent the ultimate carcinogens (Lijinsky, Frederick Cancer Research Center, USA). The effect of replacing certain hydrogen atoms in the nitrosamine by deuterium, with the consequent slowing of oxidation at this site, on the carcinogenicity of the nitrosamine gives suggestive data on the relevant reactions (Lijinsky). The effect of components of a normal diet, for example alcohol, on metabolism of nitrosamines is obviously of much relevance in assessing the role of

these compounds in human cancer (Hecht, Maylor Vana Institute, Valhalla, USA). Also the occurrence in cooked food of chemicals which increase the mutagenicity of nitroso compounds, and cause previously inactive compounds to become mutagenic, is a cause for concern (Wakabayashi, National Cancer Centre Research Institute, Tokyo).

One in four of the population develop cancer, and much of this is thought to be due to environmental agents (Higginson, International Agency for Research on Cancer, Lyon), which are, at present, mainly unidentified. The universal distribution of nitrosamines and the variety of tumours they are known to induce in animals make it difficult to correlate exposure with any particular human cancer. However, there is now epidemiological evidence that strongly suggests the association of *N*-nitroso compounds with oesophageal, bladder and stomach cancer. The fact that a variety of preventive measures are being taken to reduce exposure is a cause for special congratulation to those engaged in this work. □

Movement in membranes

from A. G. Lee

It is now ten years since Singer and Nicholson unveiled their fluid mosaic model of the biological membrane to an admiring world. To have survived so long is a complement both to the basic correctness of the model and to its flexibility. Predictably, much work over the intervening period has been concerned with trying to reduce this flexibility and achieve a state from which it is possible to make concrete predictions about membranes. Progress has, however, been slow and the gap to be bridged between simple model systems and studies of intact membranes remains large.

One source of difficulty is obvious — the membrane is a complex mixture of components, and one of the keys to success in the molecular sciences is to avoid complex mixtures. In the long run, this problem has to be faced in membrane studies, since an understanding of how the various components of the membrane mix together is one of the central issues of membrane biology. All that we can say with certainty at present is that the components of the membrane will not be mixed randomly, but the exact nature of the resulting order is unclear. Order in the membrane will follow in part from the equilibrium thermodynamic mixing properties of the components of the membrane, but it could also reflect the role of larger-scale structures in

preventing any such equilibrium mixing. The latter possibility is illustrated in a recent study by Dragsten, Blumenthal and Handler (see this issue of *Nature*, p.718).

Epithelial cells form sheets which serve as permeability barriers in organs such as kidney and the intestine. To be effective as a permeability barrier the cells have to be linked together by an elaborate junctional complex, called a tight junction, which forms a continuous, belt-like, highly impermeable structure around the cells. A further property of the cells is that they are asymmetric, with channels, pumps and receptors being different on the apical (mucosal) and basolateral surfaces. It has been suggested that this asymmetry is possible because the tight junction acts as a barrier to lateral diffusion in the membrane. Using the technique of fluorescence photobleaching, Dragsten and his colleagues have shown that cell surface sites in epithelia labelled with lectins do not diffuse laterally at all, so that no barrier is required to maintain an asymmetric distribution of these components. They also found a much more interesting result using lipid labels added to monolayers of epithelial cells. A fluorescently labelled phosphatidylcholine, for example, was free to diffuse in either the apical or basolateral surface (depending on to which side of the monolayer it was added) but was unable to pass through the tight junction from one surface to the other. However, if the epithelial monolayers were dispersed into

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suspensions of single cells by disruption of the tight junction, then phospholipid could freely diffuse over the entire cell surface.

Not all amphipathic molecules are segregated in this way by the tight junction, and some can freely diffuse from one surface of the cell to the other. These differences appear to be correlated with the ability of the label to undergo the so-called 'flip-flop' motion between the two halves of the bilayer membrane. Phospholipids are known to undergo this motion very slowly, and a phospholipid added to epithelial cells remains in the outer half of the membrane. Some amphipathic molecules can, however, rapidly flip between the two halves of the membrane, and it is these labels that can diffuse past the tight junction. It therefore seems that the tight junction acts as a diffusion barrier only for the outer half of the membrane and so allows the epithelial cell to maintain a difference in phospholipid compositions between the outer halves of the apical and basolateral surfaces of the cell.

Restricted diffusion of phospholipids and other amphipathic compounds can also result from the simple equilibrium mixing properties of the components of the membrane. One clear example has been studied by McConnell and his colleagues over the last few years (Recktenwald and McConnell *Biochemistry* 20, 4505; 1981). There is now much evidence that in mixtures of phospholipids and cholesterol some kind of highly ordered structure is formed. Although the full details are still controversial, the suggestion is that the structure resembles a ploughed field with furrows of pure phospholipid separated by ridges made up of a mixture of phospholipid and cholesterol. Diffusion will then tend to occur preferentially parallel to the ridges and furrows rather than across them. In extrapolating this conclusion to real membranes, many of which contain high concentrations of cholesterol, it is of course necessary to consider the effects that proteins might have. Effects of protein on the diffusion of amphipathic molecules are considered by Laggner in a recent issue of *Nature* (294, 373; 1981).

If spin-labelled fatty acids are incorporated into membranes at relatively high concentrations, then the ESR spectra of the spin labels become broadened as a result of motion of one spin label relative to its neighbours. Laggner has observed that this concentration broadening is less in membranes of sarcoplasmic reticulum than in lipid bilayers prepared from phospholipids extracted from sarcoplasmic reticulum. He suggested that this could reflect the presence of two phospholipid environments — bulk phospholipid and annular phospholipid adjacent to membrane proteins, with only the fatty acid incorporated into bulk phospholipid being free to diffuse within the membrane. The pattern of diffusion of amphipathic molecules in biological membranes is clearly complex. □

The gassiest comet?

from David W. Hughes

COMET BRADFIELD, 1979X, is the comet in question and recent measurements indicate that the ratio between the mass of gas and the mass of dust emitted by this comet per unit time is higher than that measured for any other comet. The underlying message is that no two comets seem to be the same and when they near the Sun their gas and dust emission depends on a whole range of factors. Among the most important of these must be the time interval between perihelion passages, the orientation of the nucleus spin axis with respect to the orbital plane and the evolutionary stage the comet happens to be in.

Knowledge of gas production has been greatly improved by two factors. First, high-resolution spectra of bright comets can be obtained from the International Ultraviolet Explorer (IUE) satellite. The satellite has two echelle UV spectrographs that have a total wavelength range of 1,150–3,200 Å and a possible resolution of 0.2 Å, over a rectangular field of view of 10×15 arc s. Data have been collected from objects as faint as 17th magnitude. Second, an upsurge in interest in the UV end of the spectrum has helped the ground-based astronomer and it is now realized that reliable, absolute photometry can be carried out from a good site down to wavelengths of around 3,080 Å.

Considerable extinction occurs in the atmosphere at low wavelengths due to Rayleigh scattering by air molecules, aerosol scattering by suspended particles and molecular absorption by ozone. Most of the ozone resides between the altitudes 10 and 35 km and the column density varies with season and latitude. Its contribution to extinction is, for example, 20 per cent smaller at the latitude of Hawaii than at the latitude of Flagstaff, Arizona. Unfortunately the extinction caused by Rayleigh and aerosol scattering varies exponentially as a function of height — for UV observations the higher you are the better. Extinction can, however, be measured by making repeated observations of a standard star as it rises and sets. The results obtained vary from 80 to 20 per cent as the wavelength increases from 3,100 to 4,100 Å.

Ground-based observations of comet Bradfield have been made by Michael F. A'Hearn (University of Maryland), Robert L. Millis (Lowell Observatory, Arizona) and Peter V. Birch (Perth Observatory, Australia) and their results have been published in a recent edition of *The Astronomical Journal* (36, 1559; 1981).

Comet Bradfield came very close to the Earth and although it was not unusually

luminous, it was possible to make detailed photometric measurements from a time just after its discovery, when it was 0.57 AU from the Sun, up to the time when it was 1.65 AU away. All observations were postperihelion. The unusual apparition geometry meant that the comet quickly changed its position on the celestial sphere and had to be observed progressively from Perth, Australia; Mauna Kea, Hawaii; and Flagstaff, Arizona. Photometric measurements were made of the comet using a series of filters with pass bands of around 100 Å. The coma of the comet contains many gas molecules which undergo fluorescence excitation by solar radiation. Filters centred on 3,085, 3,365, 3,870, 4,060, and 5,115 Å pick up the radiation from OH, NH, CN, C₃ and C₂ respectively. The dust-scattered continuum was sampled by using filters centred at wavelengths of 3,300, 3,675 and 5,240 Å.

Using a specific coma model the authors converted the observed cometary molecule column density to a value for the total number of molecules in the coma and then, by dividing by the lifetime of the species, to a value for the production rate from the nucleus. To check their work they compared their ground-based observations of the production of OH with those determined from the IUE spacecraft. The agreement was excellent.

The production rate of all molecular species was found to vary as $r^{-3.2}$ where r is the heliocentric distance of the comet. This is remarkably steep and contrasts with the r^{-2} variation found for comet West. One suggestion is that the nuclei of the two comets differ. Bradfield is perhaps covered with an outer 'frosting' of relatively volatile material. However, frosting is generally thought to be associated with a long residence (10^5 – 10^7 yr) in the boundary regions between stars, whereas comet Bradfield has an orbital period of only about 300 yr. Maybe the surface of the nucleus is heterogeneous or suffers from the buildup of an insulating crust.

The gas to dust ratio of the comet was found to be higher than for any other comet observed previously. The equivalent widths of certain C₂ Swan bands indicate that this could have been as high as 22 when the comet was about 0.8 AU from the Sun, decreasing to about 3 at 1.65 AU. This is three to four times greater than values observed for previous very gassy comets such as P/Encke and comet Kohler (1977 XIV).

Changes occurred in the relative molecular production rate as a function of heliocentric distance. The comet was CN rich when it was discovered at $r=0.57$ AU. Over the range $0.6 < r < 1.0$ AU the abundance ratios were normal and they stayed constant. However, a peak in C₂ production relative to CN occurred around 1 AU and OH and C₂ decreased relative to C₃ and CN as the comet went further away. □

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REVIEW ARTICLE

Long-term growth and cloning of non-transformed lymphocytes

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The diversity of lymphocytes has made it difficult to study the properties of a specific lymphocyte type. Here we describe methods used in long-term culture and cloning of T lymphocytes and discuss the potential uses of these cell lines in immunology.

ONE of the greatest obstacles to the understanding of the functions, regulation, cell biology and secreted products of lymphocytes is the enormous diversity of these cells. This diversity is accounted for by: (1) a series of distinct differentiation lineages, that is, B and T cells and their subpopulations; (2) a heterogeneity of differentiated states (resting, activated, memory and effector cells); and (3) a heterogeneity in the specificity of receptors individually expressed on distinct cells. The number of distinguishable specificities expressed by lymphocyte receptors is probably of the order of 10^7 . By taking into account the other two classes of heterogeneity, we may estimate conservatively that 10^8 distinct types of lymphocytes may exist among the $\sim 10^{12}$ such cells in an adult human.

Obviously, the direct study of the properties of any specific type of lymphocyte is virtually impossible in such conditions. Traditionally, immunologists have resorted to methods such as the use of 'polyclonal' stimulants which activate most or all cells of a particular class. These approaches have yielded valuable information on lymphocyte physiology but the extent to which the results obtained reflect the reality of antigen-stimulated lymphocyte function is still uncertain. Sophisticated cell purification procedures such as fluorescence-activated cell sorting and the use of antibody-coated dishes, have made it possible to obtain cell populations of a substantial degree of purity, but these procedures by themselves cannot provide truly homogeneous cell populations. Recently, however, long-term culture and cloning of non-transformed immunocompetent lymphocytes has become possible. Here we review the methods of production of such long-term T-cell lines and the potential value of these lines in cellular immunology.

Culturing

It had been possible for some time to maintain functional thymus-dependent (T) lymphocytes in culture and to enrich such cultures with cells specific for a given antigen by repetitive stimulation with that antigen. Using this approach, T lymphocytes specific for protein antigens were propagated for periods of 3 months or longer¹ and cytotoxic T-cell lines maintained for periods >60 days². However, such lines generally failed to expand to any substantial degree. It was the recognition that the growth of lymphocytes depended not only on contact with antigen but also on the presence of certain growth factors³, such as T-cell growth factor (TCGF or interleukin-2 (IL-2)), which opened the way to the large-scale growth and cloning of T lymphocytes. Various techniques are now available for developing and cloning long-term T-cell lines. Indeed, T lymphocyte lines of essentially all the major functional classes known, including helpers, amplifiers, cytotoxic cells and suppressors, have been prepared and dramatic results obtained with these lines are now being reported⁴.

Several different methods can be used to establish T lymphocytes in long-term culture. Perhaps the first, and still most widely

used, is to grow T lymphocytes from immunized donors for several weeks or more in the presence of antigen with exogenous growth factor(s), before attempting to clone them⁵. In general, T cells specific for foreign histocompatibility antigens—cytotoxic T cells and amplifier T cells—are grown with allogeneic irradiated stimulator cells whereas T cells specific for protein antigens are grown with antigen and irradiated spleen cells as a source of antigen-presented cells (APCs). A source of histocompatible APCs is particularly critical for cells displaying 'histocompatibility restriction' (see below). As well as presenting antigen, the added irradiated cells probably supply growth or differentiation factors including, but not necessarily limited to, interleukin-1 (IL-1). Cell growth is often quite vigorous in the presence of antigen, a source of an IL-2-containing growth factor and irradiated spleen cells. Cells grown in this way can often be established in long-term lines although they generally go through a period of 'crisis' when they stop growing or grow only slowly. The nature of crisis is not fully understood, but cells which survive it can be maintained in culture for periods of ≥ 3 yr (F. Fitch, personal communication). It has also been possible to establish cell lines using antigen and irradiated spleen cells, without the addition of an exogenous source of growth factor^{6,7}. This approach has been particularly successful in the establishment of helper cell lines. Presumably, in this case, IL-2 is produced by the irradiated spleen cells added to these cultures or perhaps by the line itself.

A second approach to the production of long-term T-cell lines is to grow cells as colonies in soft agar as soon as possible after they have been taken from an immunized donor⁸. In this technique, T lymphocytes are stimulated in an initial suspension culture by the addition of antigen and a source of APC, generally in the form of irradiated spleen cells. After 3 days, these cells are distributed in the upper layer of a two-layer soft agar culture system; antigen is present in the lower layer. Colonies form in the soft agar and may be picked from day 4 to day 8. These colonies are then placed in microtitre culture dishes and grown in the presence of antigen, a source of irradiated APCs and an IL-2-containing growth factor. Lines can be established from such colonies with a relatively high frequency of success.

An alternative approach is to select cells for their functional properties rather than their antigenic specificity. For example, Nabel *et al.*⁹ have obtained cells from various sources (that is, fetal liver, bone marrow, thymus and spleen) and grown them with a series of different irradiated filler cells and growth factor-containing supernatants. By analysing the cultures thus obtained, they were able to produce a series of lines of which some mediated helper function, some suppressor activity and others natural killer (NK) activity.

Cloning

Each of these strategies for the establishment of long-term lines should be followed by a cloning technique if a cell population derived from a single progenitor is desired. Indeed, it can be

anticipated that the longer the interval between the introduction of cells into culture and the time when cloning is carried out, the more likely it is that an 'unusual' cell may come to dominate a line. Consequently, it is desirable to clone as soon as possible, as in the technique described above for preparing lines from soft agar colonies.

Techniques used to clone long-term cell lines are of three general types. Perhaps the most widely used is limiting dilution cloning, in which the cells are diluted to low density (that is, 0.1 to 10 cells per well) and plated out in microtitre wells. In general, such techniques require the presence of some type of filler cell if the T cells are to grow and, for the approach to be useful, the frequency of wells which show cell growth should conform to that expected from Poisson statistics in the case of a single limiting unit responsible for cell growth. Even so, unless the cloning efficiency is quite high, it is advisable to repeat the cloning step before regarding a line as derived from a single progenitor. A second approach is to carry out soft agar colony formation in conditions where the number of antigen-presenting cells exceeds the number of the cultured cells plated. If the efficiency with which cultured T cells form colonies is high (and cases of 90% or greater have been reported¹⁰) then it is likely that progeny from such colonies are, indeed, members of a single clone. A third approach is to select a single T cell from a long-term line by micromanipulation or using a cell sorter and to grow a line from this cell. This is perhaps the most difficult but least ambiguous method.

T-cell growth factors

The preparation and study of long-term T-cell lines has produced interesting results. First, it has given great impetus to the study of factors regulating T-cell growth, two of which have been characterized in considerable detail. One of these, IL-1, is a macrophage product which seems to play an important part in the activation of some T cells, possibly by stimulating them to produce a second factor, IL-2 (refs 11, 12). Generally, the ability of IL-1 to stimulate T cells seems to depend on the presence of another stimulating agent, either antigen (or antigen-Ia complex) or mitogenic lectin. IL-1 can be obtained from macrophage tumour lines such as P388D1. Production of IL-1 by P388D1 depends on induction with agents such as phorbol myristate acetate (PMA). The technique of superinduction of P388D1 cells has recently provided sufficient IL-1, a protein molecular weight (M_r) 15,000, for it to be purified to homogeneity¹³. In addition to the role of IL-1 as a T-lymphocyte activating factor (it was originally known as LAF), it seems also to function as an endogenous pyrogen¹⁴ and as a stimulant of fibroblast proliferation¹⁵ and, thus, potentially may be responsible for much of the fibrosis seen in immune inflammation.

IL-2, a T-cell product which regulates T-cell growth, seems to act on T lymphocytes after they have been stimulated by antigen or by an antigen-Ia complex¹⁶. Recent studies¹⁷ indicate that resting T lymphocytes lack membrane receptors for IL-2 but that stimulated T cells (that is, T-cell blasts) bear ~10,000 IL-2 receptors (per cell). The binding of IL-2 to T-cell blasts appears to be critical for their completion of the cell cycle.

IL-2 has been purified both from murine and human sources and from normal lymphoid tissue and tumours. In general, IL-2 production requires stimulation of the cell; both EL-4, a murine T-cell lymphoma, and Jurkat, a human T-cell leukaemia, secrete large amounts of IL-2 on induction with PMA. Human IL-2 is a glycoprotein of M_r 15,000¹⁸; murine IL-2 has an apparent M_r of 30,000 by gel filtration^{19,20} and 23,000 by SDS-polyacrylamide gel electrophoresis²¹.

One major question concerning the regulation of T-cell growth by IL-2 is whether cells which are sensitive to IL-2 also secrete it, perhaps in different phases of their growth cycle, or whether IL-2 production and IL-2 sensitivity are always properties of independent T-cell lines. There are clear cases of IL-2-dependent lines which do not produce IL-2, for example, the cytotoxic T-cell lines available in many laboratories, while certain lines, such as those which express allogeneic mixed

lymphocyte reactivity, produce IL-2 during growth. However, there has been no clearly documented case of an IL-2-sensitive, non-transformed line which also produces IL-2, although T cell tumours with these properties have been described^{17,35}.

T-cell physiology

Cloned T-cell lines have potentially great value in the study of T-cell physiology. They have already aided our understanding of the specificity of so-called 'histocompatibility-restricted' T cells and of the nature of the immune response (Ir) gene product. In general, cells which mediate helper, amplifier or delayed hypersensitivity functions, which proliferate *in vitro* to antigen, or which act as cytotoxic effector cells, seem to recognize antigen and a product of the major histocompatibility complex (MHC)²². The MHC product recognized in these reactions is often referred to as a restriction element. For helper, amplifier, delayed hypersensitivity and proliferating T cells, the restriction element is generally an Ia antigen. This is a two-chain molecule; genes for both chains are encoded in the I region of the MHC. For cytotoxic T cells, the restriction element is usually an H-2K, D or L antigen. The study of cloned long-term T-cell lines has provided unambiguous evidence that joint recognition of antigen and restriction element is the property of a single cell and not of two collaborating cells, one specific for antigen, the other for the restriction element.

Studies of cloned long-term lines of antigen-specific human T lymphocytes have established that they, too, demonstrate histocompatibility restriction²³, a matter which had previously been in doubt for human T cells because of the difficulty in obtaining antigen-specific cells of the correct genotype and because the mixed lymphocyte reactions observed in cell mixing experiments gave confusing results.

Cloned mouse T-cell lines have been used to show that in heterozygous (F_1) individuals, restriction elements may be formed by the pairing of a product encoded by one parental haplotype and a product encoded by the other parental haplotype^{24,25}. There is chemical evidence of Ia antigens that are created by such genetic complementation²⁶. Clonal analysis suggests that clones specific for restriction elements created by complementation (heterozygous restriction elements) are only slightly less frequent than those specific for homozygous restriction elements. Indeed, the agreement between formation of both restriction elements and Ia antigens by complementation provides strong evidence that the Ia antigen is the restriction element. Similarly, using cloned T-cell lines to analyse the action of Ir genes, it has been possible to show that the products of these genes are also restriction elements and Ia antigens²⁴.

Finally, the use of cloned T-cell lines has provided insight into the provocative finding that the frequency of T lymphocytes specific for foreign MHC antigens (that is, capable of mediating mixed lymphocyte responses) is very high. It has been estimated that ~5% of the T lymphocytes of an inbred mouse are specific for the MHC antigens of any single foreign MHC haplotype. It has recently been shown²⁷ that a cloned cytotoxic T-cell line specific for an H-Y antigen and a self-MHC restriction element could also lyse cells of a specific foreign MHC type that did not express the H-Y antigen. Similarly, a clone of T cells from a mouse of MHC haplotype, $H-2^a$, which could be stimulated to proliferate by dinitrophenyl-ovalbumin (DNP-OVA) in the presence of APC-expressing $H-2^a$ -encoded restriction elements, could also be stimulated to proliferate by APC-expressing $H-2^s$ antigens in the absence of DNP-OVA²⁸. Thus, these antigen-reactive $H-2^a$ cells also mediate a mixed lymphocyte response to $H-2^s$ -encoded Ia molecules. Indeed, recent studies²⁹ suggest that a very large fraction of cloned antigen-specific, MHC-restricted T-cell lines are also responsive to foreign MHC antigens. Thus, it seems likely that a high frequency of alloreactive T cells is observed because alloreactivity may be a property of almost all MHC-restricted T cells. This will be of critical importance in understanding the chemical basis of T-cell antigenic recognition and the evolutionary forces which have shaped the T-cell repertoire.

Recently, the approaches used to grow long-term lines of T cells have also been applied to B cells. Lines of non-transformed, growth-factor dependent B lymphocytes of mouse³⁰ and human³¹ origin have been propagated for periods of >10 months. The growth of these cells seems to depend on a growth factor produced by T lymphocytes but which is distinct from IL-2 (M. Howard *et al.* and W.E.P., submitted for publication, and B.S., B. Stadler, J. J. Oppenheim and W.E.P., unpublished observations). This factor has been termed B-cell growth factor (BCGF) and studies of its properties are now in progress. B cells grown in long-term culture can be stimulated to secrete immunoglobulin and human B-cell lines have been successfully cloned by limiting dilution.

Potential

We may anticipate that the use of cloned T-lymphocyte populations will be of great value in the functional, molecular and genetic characterization of T-lymphocyte receptors and will lead

to a more complete understanding of both the molecular basis and biological significance of histocompatibility restriction. We may also anticipate that a further understanding of the regulatory interactions of immunocompetent cells will come from the preparation of cloned lines of each cell type and from the analysis of the molecules that mediate these interactions. Furthermore, the application of this technology to human T^{3,22} and B³⁰ lymphocytes will have great importance in various clinical situations. Cloned T-cell lines will be valuable as tissue-typing reagents³² and may have applications in specific therapy for certain infectious agents (J. M. Chiller, personal communication) and tumours^{33,34}.

Much work remains to be done; it is particularly important to establish the extent to which such long-term cells are 'normal' (that is, how closely they resemble lymphocytes *in vivo*). It seems reasonable to predict that long-term lines of cloned lymphocytes will become standard tools of the cellular immunologist.

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ARTICLES

The 1883 eruption of Krakatau

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The 1883 eruption of Krakatau was a modest ignimbrite-forming event. The deposits are primarily coarse-grained dacitic, non-welded ignimbrite. Large explosions produced pyroclastic flows that entered the sea, generating destructive tsunamis. Grain-size studies of the ignimbrite suggest that these explosions were not driven by magma-seawater interaction. The total bulk volume of pyroclastic deposits, including co-ignimbrite ash, is estimated to be 18–21 km³.

ALTHOUGH the paroxysmal eruption of Krakatau (or Krakatoa) in 1883 was very spectacular there are little data relating to the mechanism of the eruption, or to interpretation of the pyroclastic deposits in terms of modern volcanologic theory. Studies have concentrated on Anak Krakatau, a young cone growing in the 1883 caldera¹, and not on the 1883 deposits. The idea of caldera formation by explosive removal of cone material has been revived² but we dispute this mechanism.

We report here results mainly from new field investigations at Krakatau during September 1979. We attempt to correlate the eruption sequence reported previously with the stratigraphy of the deposits to re-evaluate proposed mechanisms for the eruption.

Most interpretations of the Krakatau event rest on the studies of Verbeek^{3,4}. He was the first to propose that the huge explosions were caused by seawater coming into contact with the

magma within the volcano. Deposits resulting from such phreatomagmatic activity for other eruptions are described elsewhere⁵. Verbeek also suggested that reaction with seawater caused the magma to froth, thus forming the extensive pumice ejected in the 1883 eruption. Although this latter interpretation is now seen to be incorrect, the question of interaction of the magma with seawater as a possible cause of the catastrophic explosions is still being debated^{2,6}. However, the eruption is usually ascribed to such phreatomagmatic activity.

Later workers have recognized that the near-source deposits of the 1883 eruption were largely emplaced by pyroclastic flows. Williams and McBirney⁷ suggested that these flows may have swept for some distance across the floor of the Sunda Straits. We now present evidence that the culminating event of the Krakatau eruption was the generation of pyroclastic flows by gravitational collapse of the eruption column after several

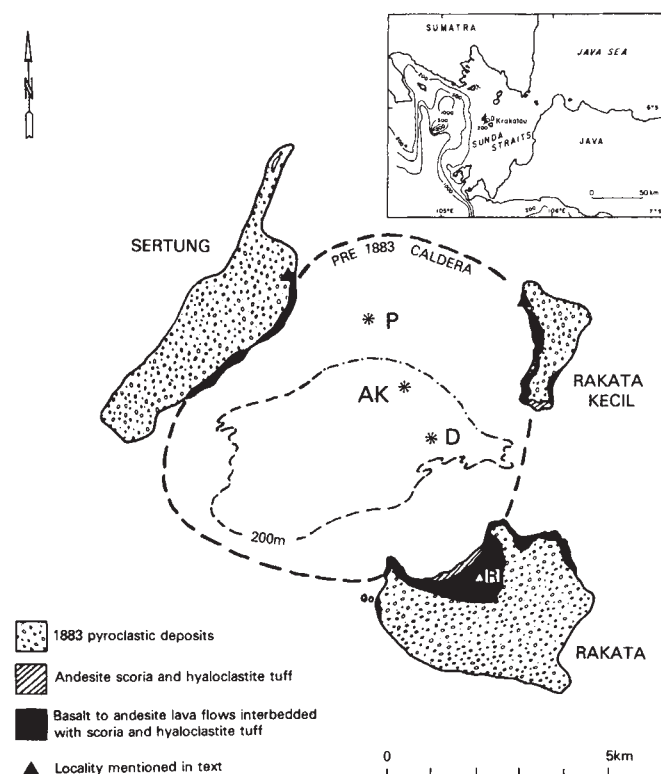


Fig. 1 Sketch map of Krakatau Islands showing prehistoric caldera, possible 1883 caldera outline (200-m isobath, lighter dashed line, dash-dot where conjectural) and simplified geology. R, Rakata cone; P and D are positions of Perbuwatan and Danan vents on pre-1883 Krakatau Island; AK, vent of Anak Krakatau. Outline of islands is from Indonesian Geological Survey map (1940). Inset: location of Krakatau Islands; isobaths in metres (after ref. 2).

large magmatic explosions. We discuss here the role of magma-seawater interaction during the eruption and suggest that the major tsunami that accompanied the eruption were produced when the pyroclastic flows entered the sea.

Before the eruption of 1883, Krakatau consisted of a large island 9 km long and 5 km wide constructed of three volcanoes, Perbuwatan, Danan and Rakata, situated along a fissure within a prehistoric caldera ~7 km in diameter⁸. The surrounding smaller islands of Sertung (formerly Verlaten Island) and Rakata Kecil (Lang Island) are remnants of the rim of the prehistoric caldera (Fig. 1). Krakatau Island largely disappeared during the 1883 eruption, leaving only the southern portion of the cone of Rakata volcano (and a small rock pinnacle) exposed above sea level. The consensus of opinion is that the main vent or vents for the 1883 eruption lay between the vents of Perbuwatan and Danan. Our results are consistent with a vent location in this general area; the location of Anak Krakatau may be controlled by the 1883 vent and conduit.

The foundation of the Krakatau volcano is largely basaltic andesite to andesite composition⁹. The 1883 eruption produced a widespread deposit of non-welded dacitic ignimbrite which covers major portions of the present Rakata, Sertung and Rakata Kecil Islands.

Stratigraphy of the 1883 deposits

We have compared the stratigraphy of the pyroclastic deposits examined in the field with the contemporary eruption records compiled by Verbeek^{3,4}, and with the later work of Stehn¹⁰ and Williams⁸. The chronology of the eruption, tsunami, stratospheric effects, and the global spread and transport of long residence time 'dust' were documented in the Krakatoa Committee Report of 1888¹¹.

A composite section of the pyroclastic deposits of the 1883 eruption is shown in Fig. 2. The stratigraphy of the airfall and

pyroclastic surge beds is based primarily on two localities on Sertung and Rakata Kecil Islands (Fig. 1), and the stratigraphy of the pyroclastic flow deposits (ignimbrite) is based on several cliff exposures on the western coasts of Rakata and Sertung Islands.

The 1883 eruption began with a period of intermittent mild explosive activity on 20 May 1883. Contemporary reports suggest that on 26 August 1883, at about 13h 00min LT the volcano went into an increasingly explosive phase of eruption, producing a more or less continuous eruption column through explosions at brief intervals (~10 min) (see Judd in ref. 11). The largest explosions during this stage of eruption were recorded at 17h 07 min on the evening of 26 August and 1h 42min, 2h 25min and 4h 43min on 27 August (Fig. 3; see Judd in ref. 11). The eruption column is reported to have been up to 25 km high during this interval. Ships within ~20 km of the volcano reported heavy ash fall, with large pumice clasts (up to ~10 cm diameter) (Judd in ref. 11).

The explosions produced fall units of dacitic pumice up to 20 m thick that are exposed on the islands of Rakata Kecil and Sertung. Pyroclastic surge deposits, found interstratified with the fall units, indicate that minor eruption column collapse during this stage of the eruption produced thin, low-density turbulent pyroclastic surges¹². All outcrops of the fall/surge deposits are very close to the source (only 2 or 3 km from the supposed vent) and it is inferred that the surges were fairly localized (with much material probably flowing onto or into the sea). The fall deposits were probably of sub-plinian type^{13,14} and also of modest dispersal. For example, there were reports of only minor ash fall in southern Sumatra and western Java before the climax of the eruption of 27 August (ref. 11, p. 14). No pumice fall deposits were found beneath pyroclastic-flow and surge deposits on Rakata Island, only 6 km south-east of the vent, even though exposure was excellent. However, the axis of dispersal of the ash fall may have been directed to the west or south-west⁴.

On Rakata Kecil Island, an estimated 3 km from the source vent, two uppermost 5–6-m thick pumice fall units are welded to form a dark-grey coherent deposit, differing markedly in colour from the over- and underlying white dacitic pumice (Fig. 4a). Sparks and Wright¹⁵ have described similar welded airfall tuffs

UNITS	COMPOSITE SECTION	THICKNESS (m)	MAJOR FLOW UNITS	LITHOLOGY
FINE AIR FALL BEDS?		2	?	Fine bedded ash
		3-15	4	Upper flow unit, approx 5m thick on Rakata to 15m thick on Sertung
IGNIMBRITE FLOW UNITS PRODUCED AFTER 5h 35m ON 27th AUGUST 1883		10	3	
		15-20	2	Coarse grained, white - grey non-welded ignimbrite, contains pumice blocks up to 1m, juvenile obsidian blocks up to 70cm and lithic blocks in a poorly sorted ash matrix. Pumice is crystal poor (9 wt%) Up to 50m exposed; Verbeek (ref. 4) reported a total of 80-100m.
		10	1	
		2-3		Lithic lag fall deposit
AIRFALL PUMICE AND PYROCLASTIC SURGE BEDS DEPOSITED FROM 13h ON 26th TO 5h ON 27th AUG		6-7		Pumice fall, incipiently welded in places
		0-2	NUMEROUS FALL UNITS	Pyroclastic surge beds, pumice and crystal-rich, cross stratified
		5-6		Pumice fall, incipiently welded
		7		Stratified fine and coarse pumice and ash-fall units; up to 12m
PRE-1883				Andesite lava flows

Fig. 2 Composite section through the 1883 pyroclastic deposits. Layers of fine ash at top of section were observed from the boat in cliff sections but were inaccessible to examination in the field.

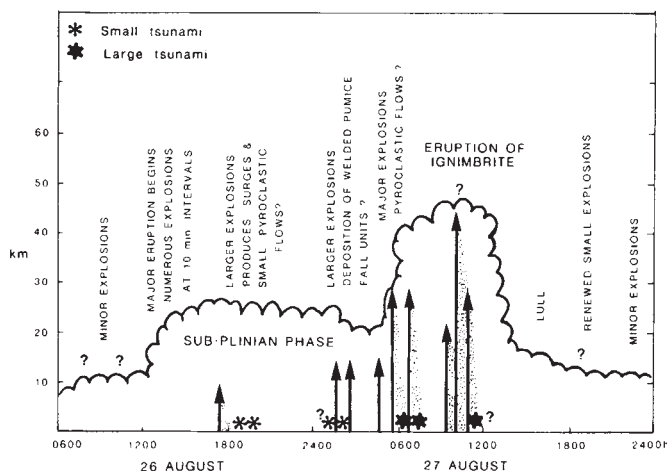


Fig. 3 Sequence of reported explosions and tsunamis during 26–27 August 1883 (all times are local). Data from refs 3, 4 and 11. Relative magnitudes of explosions, as recorded by the Batavia gasometer, are indicated by lengths of arrows. Approximate eruption column heights given at left are not accurate to more than ± 5 km. Travel times of tsunami from Krakatau to Java and Sumatra coasts estimated at between 30 min and 1 h (ref. 11).

of silicic composition; such tuffs are localized around the vent and indicate high rates of eruption and accumulation of pumice, but imply perhaps only moderate (< 2 km) heights for the gas-thrust part of the eruption column. This combination allows the hot, plastic pumice to sinter and agglutinate under its own weight on deposition. Grain-size analyses of the incipiently welded fall unit show it to be rather poorly sorted ($\sigma\phi = 3.2$), and similar to many previously studied non-welded proximal pumice-fall deposits (Fig. 5).

That the pumice-fall deposits are relatively coarse grained compared with phreatoplinian⁵ deposits (Fig. 5) suggests that, during the sub-plinian phase of the eruption, the Krakatau explosions were largely magmatic and did not involve significant interaction with seawater. The lithic-poor nature of most of the fall deposits also suggests that, during these early stages of the eruption, large-scale break-up and collapse of the volcanic edifice into the conduit (to allow access of seawater) did not occur. The surge deposits at Krakatau are also lithic poor and similar to the thin pumice-rich ground-surge deposits associated with other ignimbrite-forming eruptions and described from several other localities¹⁶.

At about 5h 30min on 27 August, the style of the eruption changed dramatically (Fig. 3). The first of five enormous explosions took place. Wharton¹¹ gives the times of the largest explosions as 5h 30min, 6h 44min, 9h 29min, 10h 02min and 10h 52min LT on 27 August; Verbeek^{3,4} gives slightly different times (3–5 min later) based on a different reading of the gasometer tracing from Batavia, which fortuitously recorded the air waves from the explosions.

We suggest that each large explosion yielded a pyroclastic flow, almost instantaneously, by large-scale gravitational collapse of the eruption column. These flows moved rapidly off the island and into the sea. The four thick major pyroclastic flow units observed in the field (Fig. 2) probably correspond to the four largest explosions (Fig. 3; see ref. 10).

Material came from the vent between explosions, but was probably finer grained and not of significant volume. We may compare this with the Mt Ngauruhoe, New Zealand eruption sequence of 19 February 1975 (ref. 17) where after an initial period of more or less continuous magma flux, activity declined, then began again with a series of large explosions, each producing a small pyroclastic flow. We propose that the Krakatau eruption sequence was similar but on a much larger scale.

Characteristics of the 1883 ignimbrite

The Krakatau pyroclastic flows apparently moved preferentially to the north and north-east and covered the islands and surrounding sea floor with dacitic ignimbrite (Fig. 6). The biased

distribution could be due to directed explosions and/or the topographical control of Rakata volcano (813 m) on the collapsing eruption column, forcing material northwards. The only reports of burns caused by hot ash and gases come from the area around Kalimbang in southern Sumatra, 40 km north-east of Krakatau and 25 km in a direct line from temporary islands produced by the pyroclastic flows. The destruction to the north may have been at the outer edge of a directed blast zone, or perhaps due to ash-cloud surges generated off the tops of moving pyroclastic flows¹². Such ash-cloud surges can apparently move across water as they did in the St Pierre disaster in 1902¹⁸.

Deposition of ignimbrite extended Sertung and Rakata Kecil Islands and the southern and eastern parts of Rakata (Fig. 6). Shallowing of the sea floor as far as 15 km to the north of Krakatau³ was caused by submarine pyroclastic flows that

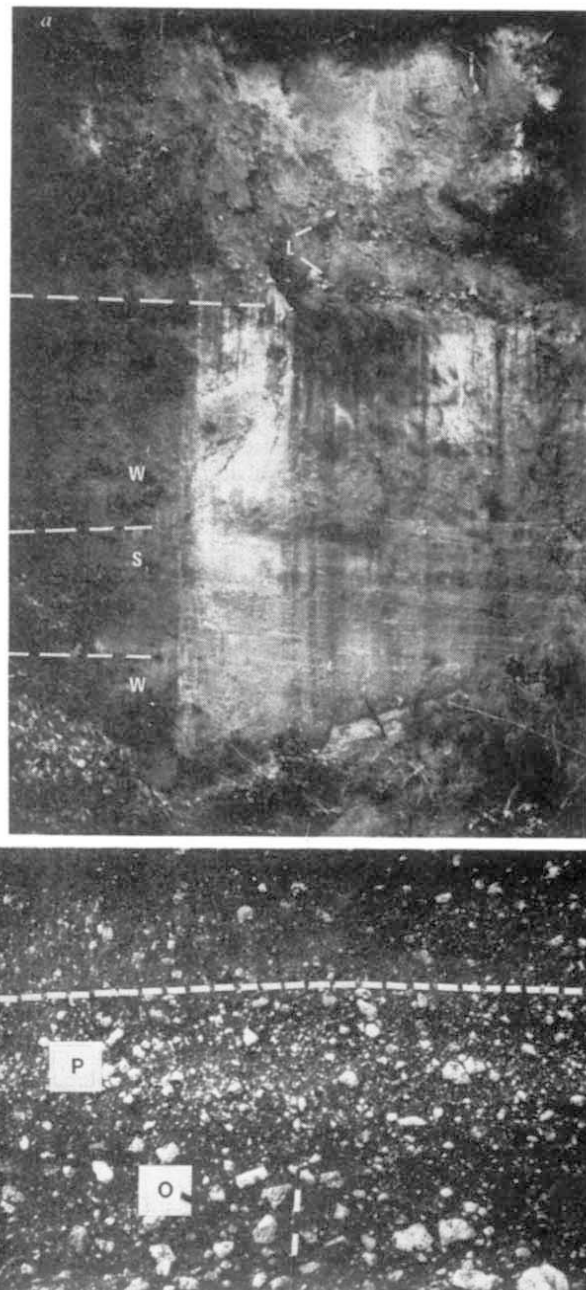


Fig. 4 *a*, Welded pumice (W), pyroclastic surge (S) and lag (L) deposits on Rakata Kecil Island at locality shown in Fig. 1. Cliff section, 15–18 m high. Above and between lag layer is 1883 ignimbrite. *b*, 1883 Ignimbrite exposed on Rakata Island. Dashed lines, flow-unit boundary; P, indicates pumice concentration at top of flow unit; O, juvenile obsidian clast. Scale is 50 cm long with 10 cm bars.

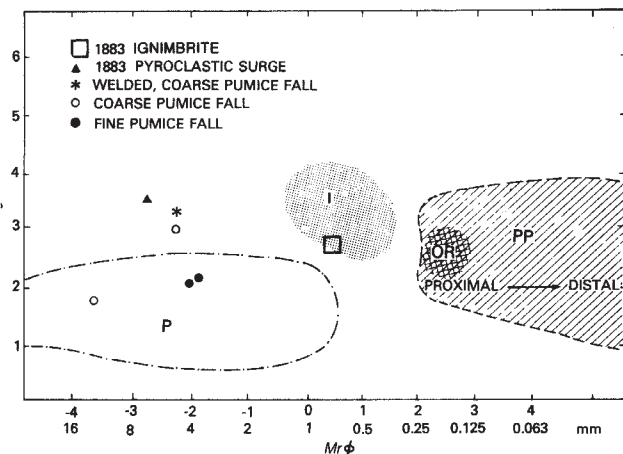


Fig. 5 Grain-size characteristics of Krakatau 1883 pyroclastic deposits, summarized on a plot of median diameter $Md\phi$ ($=\phi_{50}$) against graphical $\sigma\phi$ ($=\sigma_{84}-\sigma_{16}/2$), a sorting parameter. The Krakatau pumice-fall deposits are compared with the field of proximal plinian fall deposits (P) and the field of phreatoplinian deposits (PP), after ref. 5. The Krakatau ignimbrite (plot shows area of four analyses) is compared with the field of normal subaerial ignimbrites (layer 2b in ref. 16), (I) and that of ignimbrite produced from a phreatomagmatic eruption, the Oruanui (Or) ignimbrite (S.S., in preparation).

largely filled the 30–40-m deep basin in the sea floor; two new temporary islands, Steers and Calmeyer Islands, were portions of the ~40-m thick ignimbrite exposed above sea level (Fig. 6). Judd (ref. 11, p. 28) erroneously attributed these pumice banks to the growth of parasitic cones on the flanks of the Krakatau volcano.

Yokoyama² suggested that these temporary islands were composed primarily of lithic material derived from the explosive removal of the missing portions of Krakatau Island. However, the field descriptions of Verbeek^{3,4} and his chromolithographs of these islands⁴ clearly show them to be composed of light-coloured pumice and ash similar to the ignimbrite exposed at Krakatau. The >15 km 'run out' distance of the pyroclastic flows was a result of the vast momentum of several km³ of pyroclastic debris collapsing from a height of perhaps >5 km (ref. 19).

The 1883 magma was a sparsely porphyritic dacite with ~65–68 wt % SiO₂; the small phenocrysts are plagioclase, augite and minor opaques^{4,9}. A small amount (<5 wt %) of grey and white-streaked, mixed pumice is also present.

The ignimbrite is composed of non-welded pumice and ash with ~5 wt % lithic fragments (Figs 2 and 4b). A small but significant component (<5 wt % of the deposit) is non-vesiculated to poorly vesiculated obsidian that we consider to be juvenile. The Krakatau ignimbrite has the typical grain-size characteristics of a sub-aerially erupted ignimbrite (Fig. 5). This contrasts with fine-grained phreatoplinian deposits⁵ and associated ignimbrites (S.S., in preparation), which were apparently erupted through water. These ignimbrites are much finer grained than the Krakatau ignimbrite, especially in their lack of large-sized components near source (Fig. 5). This again indicates that magma-seawater interaction did not have a significant role in the fragmentation of the magma during the 1883 Krakatau eruption.

Initiation of ignimbrite-forming explosions

Verbeek^{3,4} suggested that the Krakatau eruption became submarine at about 10h on 27 August as a result of foundering of the volcanic edifice, and that the largest explosions were caused by seawater rushing into the vent, making contact with the hot magma and causing it to froth explosively into pumice. By contrast, Judd (in ref. 11) argued that entry of large amounts of seawater into the vent would chill the magma and cause it to crust over. Gas would then accumulate below this crust until explosive pressures were reached. These ideas both assume that

the volcano was collapsing before the explosions. However, the evidence suggests that this was not the case, and that the subsidence at Krakatau and formation of the caldera took place late in the eruption sequence.

First, lithic debris makes up only ~5% of the deposits which suggests that large-scale collapse of old cone material into the vent was not taking place during the eruption⁸. Second, the coarse-grained nature of both the pumice-fall deposits and the ignimbrite suggests that fragmentation was not caused by magma-seawater interaction. Third, the pyroclastic flow deposits that cover the southeastern part of Rakata Island, and which must have come from one of the vents to the north-west, would be cut off from their apparent source by the caldera wall collapse (Fig. 6). Thus we propose that the large pyroclastic flows were erupted before major collapse of the volcano.

The 1883 caldera lies within the prehistoric Krakatau caldera (~7 km diameter; Fig. 1), and seems to be a graben-like feature extending along two intersecting zones of fractures⁸. The 'missing' portion of the cone of Rakata may represent a large-scale slump of material into the east-west elongated caldera. The >250-m deep caldera was apparently not extensively filled with deposits of 1883 ignimbrite, also suggesting caldera collapse after the eruption of the major pyroclastic flows and emptying of the magma chamber.

We propose that seawater in large quantities did not gain access to the vent during the most explosive stages of the eruption but that seawater may have leaked slowly into the conduit area, sparking small phreatomagmatic explosions. Such explosions would have broken a cap of viscous magma and allowed sudden, explosive release of large batches of vesiculated magma from beneath the upper conduit system (see refs 20–22). Contemporary accounts indicate that the explosive activity subsided in the early hours of 27 August before the large explosions started (Fig. 3). This may have allowed a partly solidified plug to develop in the vent, facilitating the above

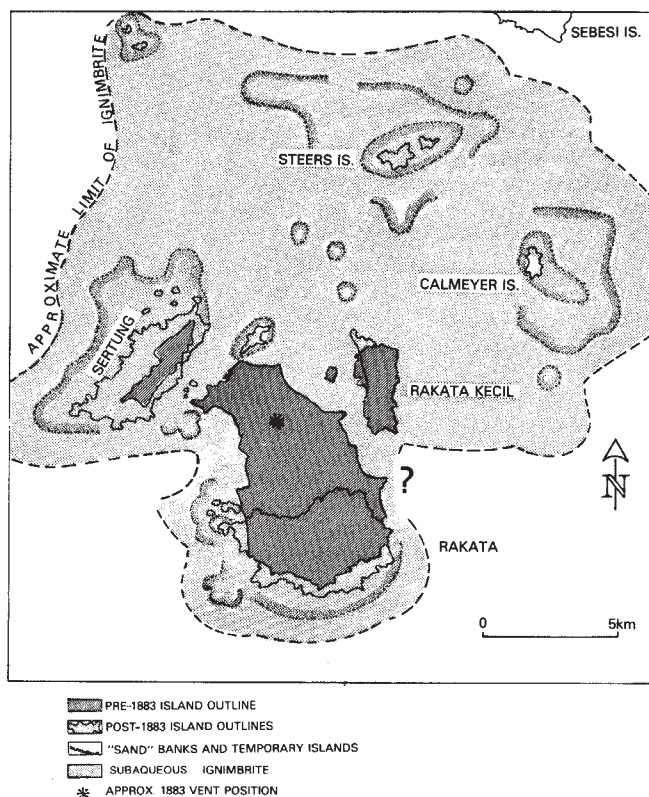


Fig. 6 Submarine distribution of the 1883 ignimbrite, inferred from maps and charts in refs 4 and 11. Hatched area shows outline of the Krakatau Islands before 1883. Stippled pattern shows extent of ignimbrite. Also shown are temporary islands and shallow banks where ignimbrite protruded above sea level (outline of islands after ref. 11).

mechanism. The presence of juvenile obsidian clasts in the ignimbrite might indicate partly solidified magma in the vent.

It has been suggested that some large volcanic explosions can be triggered by magma mixing^{23,24}. Sudden mixing of rhyolitic and basaltic or andesitic magma might lead to violent exsolution of dissolved volatiles. A small percentage (<5%) of grey and white-streaked pumice occurs in the Krakatau ignimbrite, suggesting some mixing of magmas. However, we cannot envision a magma-mixing process that would adequately explain the sequence and timing of the large explosions of Krakatau as alluded to by Rice²⁵. Magma mixing may have had a role in initiating the eruption, but we do not believe that mixing events were the prime cause of the major explosions on 27 August.

Co-ignimbrite ash

During the eruption of the pyroclastic flows, much vitric dust was dispersed in the atmosphere and formed a widespread thin, fine ash-fall deposit⁴. Considerable distal ash fall did not occur until the large explosions of 27 August, and we suggest that the widespread ash fall was mainly co-ignimbrite ash²⁶.

Mechanisms envisaged for the generation of the high-altitude column of fine vitric dust and gases that accompanies many events involving production of pyroclastic flows are: (1) rise of selectively fine (low terminal fall velocity) material above the vent; (2) generation of ash from the moving pyroclastic flows; and (3) secondary explosions that may occur as hot pyroclastic flows enter the sea⁶. Evidence for this type of secondary explosion was not found at Krakatau, possibly because of the lack of lateral exposure. However, the expected airfall layers produced by such secondary explosions are not present between the ignimbrite flow units at Krakatau in the outcrops we examined.

All large ignimbrite eruptions are accompanied by the production of co-ignimbrite ash²⁶. This fine ash consists of the vitric component—pumice shards and fragmented shards commonly from 50 μm down to a few μm in diameter. The finest ash was capable of significant stratospheric residence times varying from days to months (depending on size). This dust may have been partly responsible for the widespread atmospheric optical phenomena observed after the August 1883 eruption (refs 27, 28, Russell and Archibald in ref. 11), although the sulphate aerosols generated by the eruption were probably more important²⁹.

Volume of the 1883 eruption

Calculations of the total volume of ejecta from the 1883 eruption must include the volume of widely dispersed dust and ash as well as the subaqueous ignimbrite. Volumetric estimates range widely from Verbeek's 18 km³ for the total ejecta^{3,4}, to the Royal Society's value of 14.4 km³ for the fine distal ash fall alone¹¹, to recent estimates of 13 ± 4 km³ (ref. 2) and only 5 km³ (ref. 30) for the total ejecta based on the volume of the caldera.

Close to Krakatau, Verbeek⁴ estimated from field investigations and depth soundings of the sea bottom before and after the eruption that 12 km³ (bulk volume) was ejected. This is a reasonable figure for the volume of ignimbrite produced, based on a sheet of ignimbrite averaging ~ 40 m thick and covering an area of ~ 300 km² (Fig. 6). Verbeek also considered the volume of widely dispersed ash and estimated this to be 6 km³. This value must be a minimum because it only considers a portion of the total dispersal area, which the Krakatau Committee estimated to be 1.1×10^6 miles² (see ref. 11, p. 448). Our recalculation of the volume of widely dispersed ash from data in ref. 4, using an area against thickness plot and extrapolating to 1 mm thickness³¹, gives 8.5 km³. Recent studies of co-ignimbrite ash volumes compared with the volume of the parent ignimbrite indicate that up to 50% of the magma erupted can be co-ignimbrite ash^{26,32}. Applying the methods for estimating crystal concentration in ignimbrites^{26,33}, preliminary studies suggest that the Krakatau ignimbrite must have lost at least 40% of the vitric component into the fine co-ignimbrite ash, which there-

fore has a minimum volume of ~ 5 km³. The above calculations suggest a bulk volume of fine, dispersed ash between ~ 5 km³ and 8.5 km³.

The pre-27 August sub-plinian fall and surge deposits are thought to be of small volume, probably < 1 km³. Analysis of samples suggests that only 8% of fine vitric material in these deposits is missing. Therefore, the volume of fine ash produced during the sub-plinian phase of the eruption is very small compared with that emitted during the ignimbrite-forming phase. When the amounts of ignimbrite, co-ignimbrite ash and sub-plinian deposits are added up, the total bulk volume of the 1883 Krakatau deposits is 18–21 km³. The equivalent volume of dense rock may be roughly estimated as 9–10 km³.

Generation of tsunami

The Krakatau eruption also produced tsunami that inundated coastal areas around the Sunda Straits. These tsunami are generally attributed to submarine explosions or to caldera collapse^{8,34} although ejecta falling into the sea has also been suggested^{3,4,11}. The evidence indicates that the tsunami were caused by several cubic kilometres of pyroclastic flow material entering into the sea immediately after each of the large explosions.

The early small explosions of 26 August were followed by small tsunami (Fig. 3). For example, an explosion occurred at about 17h 20min on 26 August, and the first destructive waves reached the Java and Sumatra shores 40 km from Krakatau soon afterwards, between 18h and 19h. This early wave or waves may have been due to surges or small pyroclastic flows entering the sea. No other destructive waves were observed until the morning of 27 August (Wharton, in ref. 11). At about 6h 30min a large wave swept over much of the Java coast; another wave followed at about 7h 30min. At about the same time a wave hit low-lying areas of Sumatra. These waves followed the large explosions at 5h 30min and 6h 44min (Fig. 3).

At some time after 10h a gigantic wave (or waves) inundated the coasts bordering the Sunda Straits. This wave is reported to have been the largest, and was the last recorded in the straits; most survivors of the earlier waves had by this time fled from the coastal areas. The tide gauge at Batavia (Jakarta) also recorded waves that correspond to the large explosions.

The sequence and timing of tsunami suggest that the major tsunami were generated at the times of the explosions (Wharton, in ref. 11). Pyroclastic flows resulting from column collapse would have entered the sea within about ~ 30 s of the explosions¹⁹ and initiated the tsunami. The slumping of large parts of the volcano, for example the segment of Rakata cone that probably slid into the caldera, could have been a factor in generating tsunami late in the eruption.

The idea that tsunami can be caused by pyroclastic flows entering the sea is supported by a study of the huge eruption of Tambora in 1815 during which tsunami were generated which flooded the nearby coasts of Sumbawa to a height of 4 m, and Java to a height of 2 m (ref. 33). These waves could not have been produced by magma-seawater interactions or caldera collapse, as the volcanic crater lies some 15–20 km inland at an elevation of about 2,850 m. Pyroclastic-flow deposits from the 1815 eruption entered the sea at the base of the volcano (M.R.R. and S.S., in preparation), and this was the most likely cause. The large tsunami that are inferred to have accompanied the Minoan eruption of Santorini³⁴ may have been generated in the same way.

We conclude that the 1883 eruption was an ignimbrite-forming event of modest volume. The deposits are largely coarse-grained non-welded dacitic ignimbrite and show little support for a phreatomagmatic origin. The occurrence of welded pumice-fall deposits beneath the ignimbrite also seems to argue against water-magma interaction in the early stages of the eruption. The presence of mixed pumice clasts indicates some magma mixing and supports the contention that welded airfall deposits result, in some cases, from superheating of

magma during a mixing event¹⁵.

During the paroxysmal phase, four or five large-volume explosions driven by magmatic gases led to collapse of the eruption column, and to the production of pyroclastic flows that entered the sea. The blasts and ensuing pyroclastic flows seem to have been directed preferentially towards the north and north-east. The times of the explosions correlate with the times of tsunami generation, and we propose that the entry of the pyroclastic flows into the sea caused the major tsunami. Caldera collapse probably came late in the eruption.

The total volume of the deposits of the Krakatau eruption is estimated to be 18–21 km³ (bulk volume), including 12 km³ of

ignimbrite and up to 8.5 km³ of distal co-ignimbrite ash fall. When compared with prehistoric ignimbrite-forming events, ranging in volume up to 10³ km³ (refs 35, 36), the volume of the Krakatau eruption was very modest.

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Evaluation of the Seasat wind scatterometer

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Surface wind velocities have been derived from backscatter measurements of the ocean surface made by a satellite-borne, microwave sensor. Comparisons with high-quality surface-based measurements obtained during the Joint Air-Sea Interaction experiment are described. The accuracy of the scatterometer winds at this mid-latitude site, $\pm 1.6 \text{ m s}^{-1}$ in speed and $\pm 18^\circ$ in direction, for winds between 3 and 16 m s^{-1} is within the design specification.

THE Seasat-A Satellite Scatterometer (SASS) was one of several microwave remote sensors onboard the first oceanographic satellite¹, Seasat-A, and provided wind velocity estimates over the world's oceans from a height of 800 km. The technique is based on the sensitivity of microwave radar backscatter to the amplitude of short gravity waves (few centimetres) created on the sea-surface by the action of the wind. The sensor and the algorithms used to convert radar backscatter into winds are detailed elsewhere^{2–5}.

Design specifications for the SASS required a wind speed measurement range of 4–26 m s⁻¹ with an accuracy of $\pm 2 \text{ m s}^{-1}$ or 10% (whichever is the greater) and a wind direction range of 0–360° with an accuracy of $\pm 20^\circ$. To check whether these criteria are satisfied the inferred wind vectors must be compared with those obtained by surface instrumentation. Because the accuracy and coverage of routine wind reports at sea are generally not high enough to evaluate the scatterometer measurements properly, a special series of measurements from ships and buoys was conducted in the Gulf of Alaska⁶. It was also fortunate that the field phase of the Joint Air-Sea Interaction (JASIN) experiment⁷ took place during the lifetime of Seasat. One of its aims was to provide accurate estimates of the surface meteorological fields over a 200 × 200 km area, and, as a result of careful intercalibrations, high-quality surface wind data are therefore available. This article describes the results of

comparisons made at the Seasat-JASIN workshop⁸ and demonstrates that the specifications for the SASS have been met for wind speeds up to 16 m s⁻¹.

Seasat scatterometer

The strength of the radar backscatter (normalized radar cross-section σ^0) is a function of the amplitude of very short gravity waves which is itself proportional to the near-surface wind speed. Further, the radar backscatter is anisotropic, so that wind direction can be derived from scatterometer measurements at different azimuths.

The SASS was designed to view the ocean at two azimuths, as shown in Fig. 1. Four dual-polarized, fan-beam antennas were aligned such that they pointed $\pm 45^\circ$ and $\pm 135^\circ$ in azimuth relative to the subsatellite track to produce an X-shaped pattern of illumination on the Earth. In this way, a given surface location was viewed first by the forward antenna and then ~1–3 min later (depending on whether the location was at the inner or outer portion of the swath) it was viewed orthogonally by the aft beam. Twelve Doppler filters were used to subdivide the antenna footprint electronically into resolution cells ~15 km (across beam) × 70 km (along beam). In the results presented here pairs of cells were selected having separations <37 km. It is assumed that during the interval between samples the short gravity-wave field integrated over such areas remains constant. The incidence

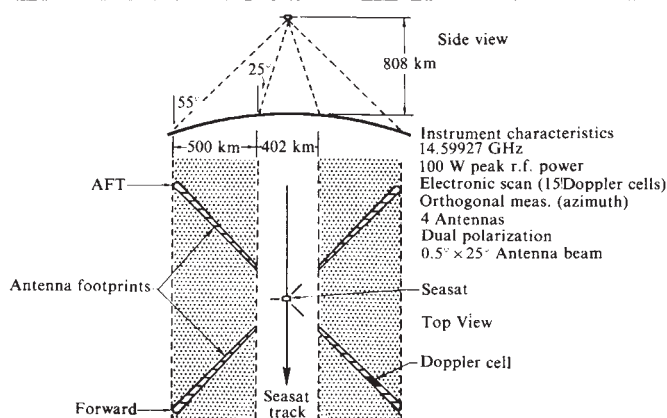


Fig. 1 The swath geometry of the Seasat Scatterometer: viewed from the side (upper) and from the top (lower).

angles of the cells ranged from 25° to 55° for the region of forward and aft beam overlap. Three additional measurements from incidence angles near nadir covered the subtrack, although no directional information can be obtained in this case.

The SASS geophysical algorithm³ enables wind speed and direction at the sea surface to be computed from σ° measurements after correction for attenuation through the atmosphere. This arises principally from water vapour, the vertical integral of which was estimated using data collected by a microwave radiometer on the same satellite⁹.

The model function $F(W, \chi)$ is the empirical relationship used to describe the dependence of the ocean σ° on the wind vector

$$F(W, \chi) = G(\theta_i, \phi, \epsilon) + H(\theta_i, \phi, \epsilon) \log W$$

where θ_i is the incidence angle, ϕ is the azimuth angle between the wind direction and the radar look angle, ϵ is the polarization of the beam and W, χ are the wind speed and direction. Several model functions have been used in the past having different values of G and H for the various combinations of θ_i, ϕ and ϵ . The coefficients were obtained from near-simultaneous measurements of σ° and surface wind speed and direction. An airborne scatterometer^{10,11} provided 3,000 σ° values and 2,000 more resulted from SASS during GOASEX⁶. A least-squares method is used to find values of W and χ that, for a given model function, will produce the best fit to the σ° observations. Two to four wind vectors result which are approximately equal in magnitude but are widely separated in direction. These multiple local minima (aliases) are due to the harmonic dependence of σ° on wind direction. The solutions are not random and it has been shown¹² that synoptic meteorologists can, using SASS data alone, select the direction closest to the observed wind on a high proportion of occasions. At the time of the Seasat-JASIN workshop the SASS data still contained the aliases so statistics were computed using the solution which gave closest agreement with observations.

Before the workshop, two model functions (W-7 and CWK)

Table 1 Summary of statistics for SASS compared with surface wind field data averaged over all incidence angles

	CWK		
	H	V	Both
Speed (m s^{-1})	-0.44 ± 1.58	0.50 ± 1.26	-0.04 ± 1.41
Direction ($^\circ$)	1.2 ± 17.9	3.4 ± 15.6	0.9 ± 17.1
No.	317	336	704
	W-7		
	H	V	Both
Speed (m s^{-1})	0.40 ± 1.61	0.84 ± 1.60	0.62 ± 1.55
Direction ($^\circ$)	-0.4 ± 18.5	1.5 ± 16.6	-0.6 ± 17.1
No.	317	336	704

Statistics for both model functions and different polarizations are shown.

had been developed using the aircraft and GOASEX data sets and were used to generate two sets of SASS wind data for 23 overpasses, chosen to give good spatial coverage of the JASIN area and as wide a range of wind speeds as possible. The JASIN surface data were withheld from algorithm developers and thereby provided the first independent assessment of the scatterometer's wind-measuring capabilities.

The JASIN experiment

During JASIN, 14 ships, 3 aircraft and 35 surface and subsurface moorings were deployed in an area 200 km off north-west Scotland during July to September 1978 and several of these platforms were equipped with wind-measuring devices. Three ships, stationed at the vertices of a 200-km triangle, reported winds every hour with a fourth ship at the centre for some of the time. Two other roving ships and several buoys near two of the corners carried data logging systems. Careful intercomparisons were made between the ships and buoys to produce an internally-consistent data set in which interplatform differences in winds are $<0.5 \text{ m s}^{-1}$ and 5° . The absolute accuracy of the winds is difficult to ascertain but both wind-tunnel calibrations and momentum flux comparisons with aircraft suggest that this error is 2 or 3%.

Wind speeds experienced during the 23 Seasat overpasses ranged from 0 to 16 m s^{-1} with 83% distributed between 6 and 14 m s^{-1} . Measurements were made at heights from 2.5 to 23 m above the surface and in different atmospheric stabilities. In this layer there is considerable variation of speed with height due to friction (direction being constant) so all measurements were corrected to give the 19.5-m neutral stability wind. This is the wind speed that would result at 19.5 m from a given surface stress if the atmosphere were neutrally stratified with an adiabatic lapse rate. The SASS algorithm also outputs the 19.5-m neutral stability wind as it has no means of estimating the stability.

Comparison of SASS winds with surface-based data

First, SASS winds were compared with autologged data from ships and buoys within the JASIN triangle. The latter provided

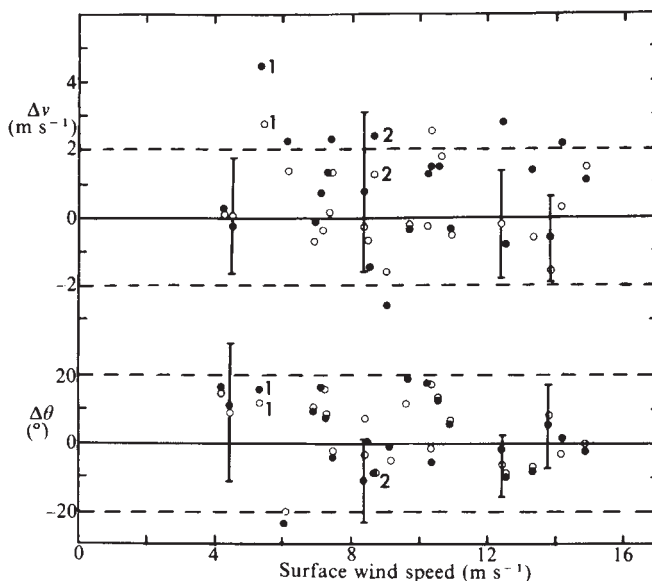


Fig. 2 SASS wind minus surface wind (speed Δv and direction $\Delta \theta$) as a function of surface wind speed. The surface winds are 60-min averages of logged data centred on the time of the satellite pass. All winds have been corrected to 19.5 m. Each point represents the mean difference for all comparisons on a particular overpass (total of 1,150 on the 23 passes). ●, W-7 model; ○, CWK. In both cases vertical and horizontal polarizations have been combined. Selected standard deviations are shown for clarity; two cases had much higher standard deviations ($>3.5 \text{ m s}^{-1}$) and are annotated.

time series of 1–15-min means which were combined to form 60-min means centred on the time at which the satellite pass occurred. This procedure is an attempt to obtain winds which are consistent with the areal average of the SASS. Figure 2 shows differences between SASS and surface winds for both model functions expressed as a function of wind speed. Most of the points lie within the design specifications. The two numbered points refer to different occasions when the differences exhibited large scatter. The more obvious was Case 1 with a mean difference of 4.5 m s^{-1} and a standard deviation of 4.7 m s^{-1} . For this case, very high SASS winds were indicated having values up to 22 m s^{-1} in a $4\text{--}5 \text{ m s}^{-1}$ field. The anomaly was localized and from ship reports and data from the microwave radiometer subsequently identified with a thunderstorm¹³. For Case 2 the surface data revealed that the comparisons were made in an area of horizontal wind gradient ahead of a cold front, which accounts for much of the observed differences, the SASS footprints being some distance from surface platforms.

It is reasonable to assume that in all cases some of the scatter is due to real spatial variability in the winds which is not satisfactorily removed by time-averaging the surface data. An attempt to smooth such effects out was made by constructing wind fields showing isotachs and streamlines based not only on

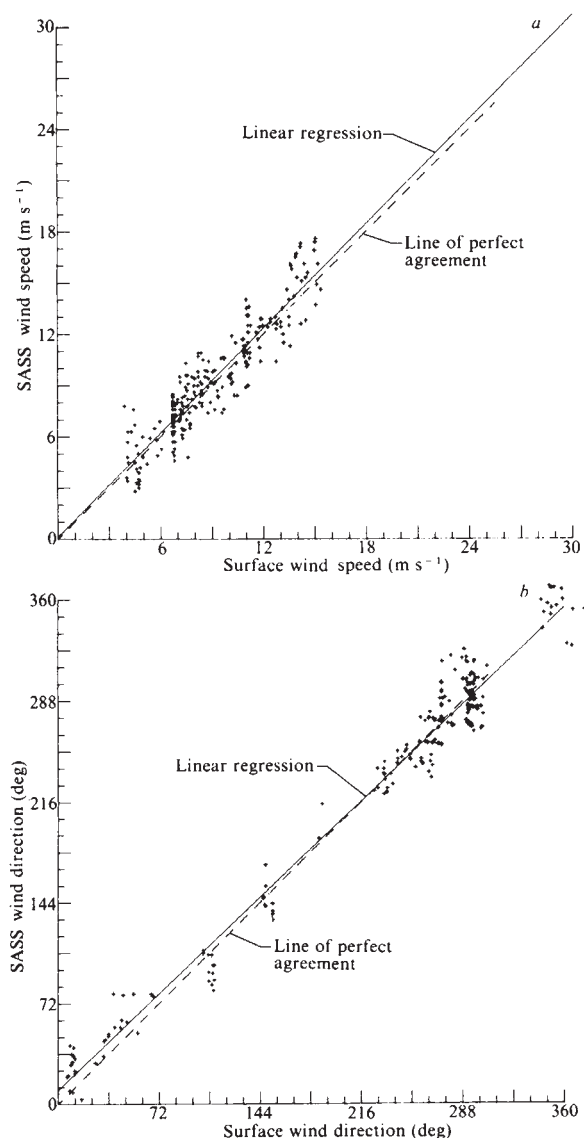


Fig. 3 Scatter plot of: *a*, SASS wind speed versus surface wind speed for CWK model; *b*, SASS wind direction versus surface wind direction. The surface data are taken from smoothed fields based on autologged data and manual observations. Slopes: 1.0195 (*a*); 0.9564 (*b*). Intercepts: 0.1094 (*a*); 10.0360 (*b*). Correlation coefficients: 0.9200 (*a*); 0.9872 (*b*).

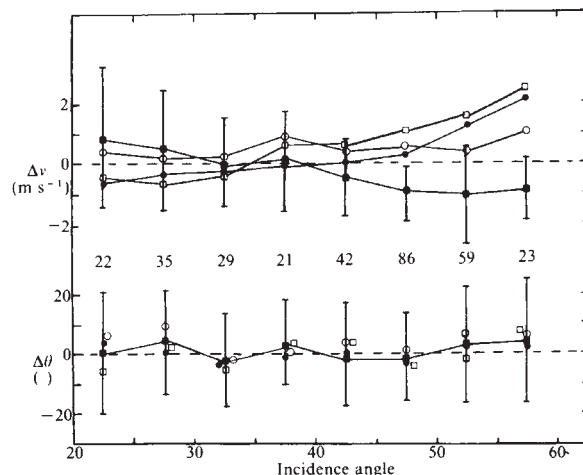


Fig. 4 Comparison of SASS with surface winds derived from plotted fields for various ranges of incidence angle. Horizontal and vertical polarizations for the two model functions are shown. For the CWK, H-pol case the standard deviation of the individual comparisons is also shown together with the number of measurements in each category. Those for the other cases are similar. ■, CWK, H-pol; ○, CWK, V-pol; ●, W-7, H-pol; □, W-7, V-pol.

the autologged data but on hourly manual observations from the corner ships to increase the spatial coverage. Values of winds were interpolated at 0.5° latitude and longitude grid points and were available for collocation with SASS data points. A cross-check of these data with the *in situ* observations discussed previously showed agreement to $\pm 0.6 \text{ m s}^{-1}$ and $\pm 4^\circ$.

Statistics were again calculated using the solution with wind direction closest to the observed value. Scatter diagrams of SASS wind speed and direction (CWK model only) versus surface measurements are shown in Fig. 3 and their dependence on incidence angle and polarization is shown for both W-7 and CWK model functions in Fig. 4. For several categories the standard deviation slightly exceeds the design specification; however, note that for these cases the statistical uncertainty is large because of the limited sample size. These figures illustrate the differences between SASS and JASIN smoothed winds, only a small part of which can be attributed to errors in the JASIN data.

Conclusions

One objective of the Seasat-JASIN workshop was to demonstrate that winds near the sea surface could be inferred to an accuracy of $\pm 2 \text{ m s}^{-1}$ in speed and $\pm 20^\circ$ in direction by means of SASS without prior knowledge of the surface data. In wind speeds up to 16 m s^{-1} the SASS wind vector algorithm recovers wind speed to an accuracy of $\pm 1.6 \text{ m s}^{-1}$ for W-7, $\pm 1.4 \text{ m s}^{-1}$ for CWK and wind direction to an accuracy of $\pm 18^\circ$ for both model functions (see summary in Table 1). Differences between the two are small and are most noticeable in the dependence of SASS wind speed on incidence angle. The worst cases occur with incidence angles below 25° and above 55° as also found by Halberstam¹⁴ in a comparison with the combined GOASEX/JASIN surface data sets.

In this study the surface data were far superior in accuracy and sampling to data provided by the routine meteorological network. They were also of higher quality than data from GOASEX in that careful analysis of the many intercomparisons between JASIN platforms resulted in reduction of the biases which were present. This in turn has reduced the scatter in comparisons with SASS^{15,16}. The scatterometer data are now being used in combination with the JASIN data to increase our understanding of how the atmosphere and ocean interact on a variety of time and space scales.

We thank the JASIN investigators for initial versions of the surface meteorological data.

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Pontnewydd Cave in Wales—a new Middle Pleistocene hominid site

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An Acheulian industry in association with a hominid molar has been found at Pontnewydd Cave. This tooth represents the oldest hominid specimen known from Wales and, except for the Swanscombe fossil, from Britain. The molar is probably from a young adult and the tooth closely resembles those of 'Early Neanderthal' fossils from Krapina, Yugoslavia, a comparison accentuated by the degree of taurodontism at both sites. Uranium–thorium and thermoluminescent dates suggest an age for the specimen of around 200 kyr. Uranium relative dating indicates a Pleistocene age for two additional unstratified hominid fragments. Sedimentological study of the deposits and examination of the rock types in the cave, both natural and artefactual, indicates earlier glacial activity.

PONTNEWYDD CAVE has been the subject of four seasons of excavation (directed by H. S. Green for the National Museum of Wales). Excavation of the cave in the 1870s by Boyd Dawkins^{1,2} and by McKenny Hughes^{3,4} produced fauna, including a possible human tooth, since lost, and artefacts which were compared with finds from Le Moustier and St Acheul. No further work took place at the cave until the present excavation except for construction work at its mouth during the Second World War, when the cave was converted to a store.

Location and geomorphological setting

The cave is situated in the lower Elwy valley of North Wales (Fig. 1), the entrance opening off the Carboniferous Limestone scarp that forms one side of the valley. The cave entrance is at a height of 89.5 m OD, exactly 50 m above the river Elwy. The valley contains thick deposits of drift, in places built into a series of terraces whose upper surface locally approximates to the height of the cave. The data from the cave should elucidate the Quaternary geology and geomorphology of the area for which, hitherto, there has been no reliable chronological framework in which to place the Quaternary sequence. Moreover, as the cave is situated close to the probable maximum limit of penetration of Irish Sea Ice into the area, it may provide a means of dating the advances of this ice sheet as well as those of the more local Welsh Ice.

Stratigraphy

Sedimentological studies were originally aimed to correlate the sequence of the three separate areas of the cave in which substantial deposits survive intact (Fig. 2a: South Fissure and

adjacent Deep Sounding; South Passage; and East Passage). High probability correlation, based on lithologic characters, has resulted in the composite stratigraphy shown in Fig. 2b. Interpretation has proceeded using data derived from both standard sedimentological analyses and the study of microtextural assemblages of quartzitic particles by scanning electron microscopy (SEM).

The lowest stratigraphical unit, the Basal Sands and Gravels, consists of a complicated series of deposits containing matrix-supported, well rounded siliceous particles, frequently 100–150 mm in diameter. These pebbles are often crudely oriented in field exposures, suggesting *en masse* movement. Discrete lenses of generally matrix-poor, finer-grained material (coarse sands and fine gravels) are common throughout the deposits, both vertically and laterally, as are lenses of laminated clayey silts. Conditions of periodic streamflow, ponding and gentle slumping, as a result of mudflow processes, are indicated. SEM examination suggests that the silt and sand-sized material from all these deposits originates from a mixed provenance and an equally mixed range of environments of modification. The sands are probably derived from local tills and fluvial sediments, introduced into the cave after a greater or lesser mixing of the original sources.

The Intermediate Complex comprises a group of deposits that are lithologically transitional between the Basal Sands and Gravels below and the Breccias (infra) above. In general terms, the Complex may be characterized by the presence of siliceous pebbles, badly sorted coarse sand and highly altered limestone clasts. Internal stratigraphical relationships between different areas are unclear, due to the discontinuous nature of individual

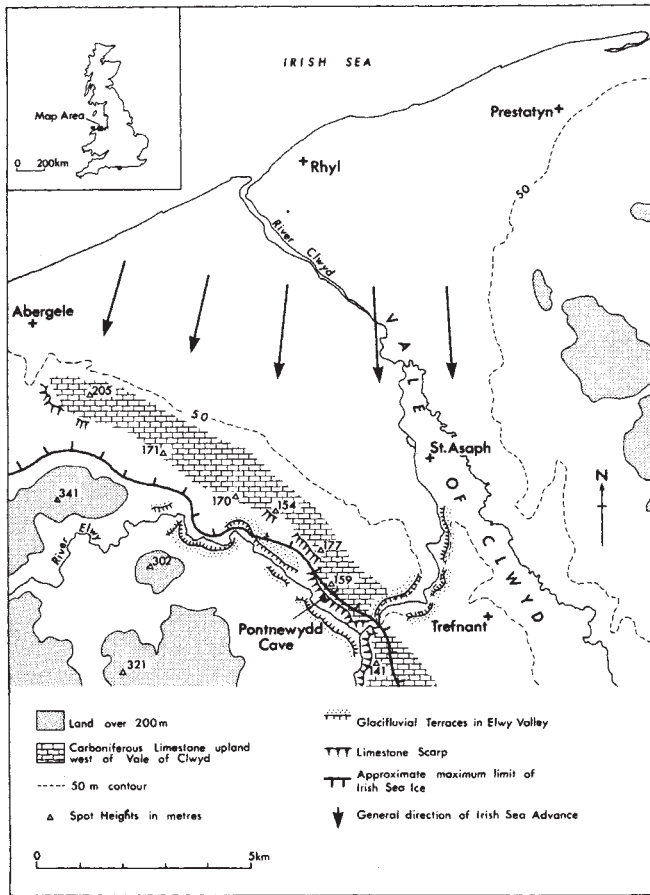


Fig. 1 Pontnewydd Cave. Location and geomorphological setting.

lenses and extreme lateral variability. Some occurrences show the combined effect of syngenetic growth of carbonate concretions and input of large quantities of organic matter. Microscopically, the grain textural suite combines qualities of both the underlying and overlying deposits, although the grain surface textural modification is basically fluvial in origin. Nowhere are primary fluvial deposits clearly evidenced; the matrix-supported fabric and poor sorting indicate that mass movement was again the last major emplacement process involved.

The two units of the Breccias comprise many angular coarse particles, mainly limestone, with medium to strong carbonate cement. Compared with the Lower Breccia, the Upper Breccia contains much more silt and fine sand, fewer but more severely fractured siliceous pebbles, and abundant but considerably less altered limestone clasts. The units are separated by stalagmite growth and laminated silts in some places. Under SEM, the Breccias are distinctly different from the Basal Sands and Gravels, although they show similar intra-sample variation. There are either provenance variations or process modification differences, or both, between the two Breccias. Whilst both contain mainly mechanically crushed quartz grains, surface modification, of only limited extent in both cases, is primarily fluvial in the Lower Breccia but both fluvial and diagenetic in the Upper Breccia. These deposits are the result of mudflow on a major scale, originating from the direction of the cave entrance. This process was interrupted once, during a period of relative quiescence, by limited fluvial (local drainage) and pool sedimentation, together with the growth of stalagmites, in what was probably a closed cave environment. The Breccias incorporate reworked material from the underlying deposits and, at one point, the Upper Breccia has clearly channelled the Lower Breccia and Intermediate Complex; this is consistent with the mode of emplacement envisaged. The limestone component and some of the silts and fine sand result from repeated inputs of entrance facies sediments. Alteration and cementation of the Breccias occurred before the entry of the next deposit.

There are no recognizable stable surfaces within the Breccias or the Intermediate Complex. Artefacts and fauna, which occur throughout the former and in certain deposits of the latter, would have been subject to transport and reworking like any other particle during mass movement.

In the South Fissure only, the Breccias are overlain by a small remnant of uncemented, silty Red Cave-Earth, with abundant angular limestone clasts. These clasts lack all signs of chemical alteration but are slightly edge-rounded, indicating derivation from unweathered entrance facies sediments by mass movement. The quartz grains are texturally different from those of the Breccias but further analysis is necessary before any interpretation can be made.

A series of current-bedded clays and sands is present in all areas, overlying either the Red Cave-Earth or the Breccias and is the result of an effluent stream that is clearing a choked drainage system. This unit (the upper clay and sands) is followed conformably by a thick stalagmitic floor.

Table 1 Comparison of measurements on the Pontnewydd molar and M² of other Pleistocene hominids last glaciation Neanderthal sample recomputed from data in ref. 33.

Measurement	Pontnewydd 1	<i>H. erectus</i> (Peking) ¹⁷			European middle Pleistocene ^{20,21}			Krapina ²³			Last glaciation Neanderthal			Early Upper Palaeolithic ³³		
		Mean	s.d.	<i>n</i>	Mean	s.d.	<i>n</i>	Mean	s.d.	<i>n</i>	Mean	s.d.	<i>n</i>	Mean	s.d.	<i>n</i>
Length (mesio-distal)	10.9 (11.2 allowing for mesial wear)	10.9	0.7	7	11.9	0.3	3	11.3	0.4	14	10.6	0.7	12	10.6	0.8	9
Breadth (bucco-lingual)	12.9	12.7	0.5	7	13.5	0.4	3	12.8	0.2	14	12.9	1.2	12	12.1	0.7	9
Crude crown area (1 × b)	140.6–144.5	138.6	8.0	7	160.6	5.3	3	144.3	7.2	14	136.8	17.0	12	126.8	15.9	8
Root height	15.8 (lingual)	14.6	1.6	7	‘Modern man’ ¹⁷ 12.6–13.6											
Root length (m – d)	9.5 (preserved portion only)	8.1	0.6	5												
Root breadth (b – 1)	12.3 (preserved portion only)	12.2	0.6	5												
Root rubusticity (1 × b)	116.9 (preserved portion only)	98.3	11.9	5	79											

Standard deviations (s.d.) and sample size (n) as indicated. Measurements in mm or mm².

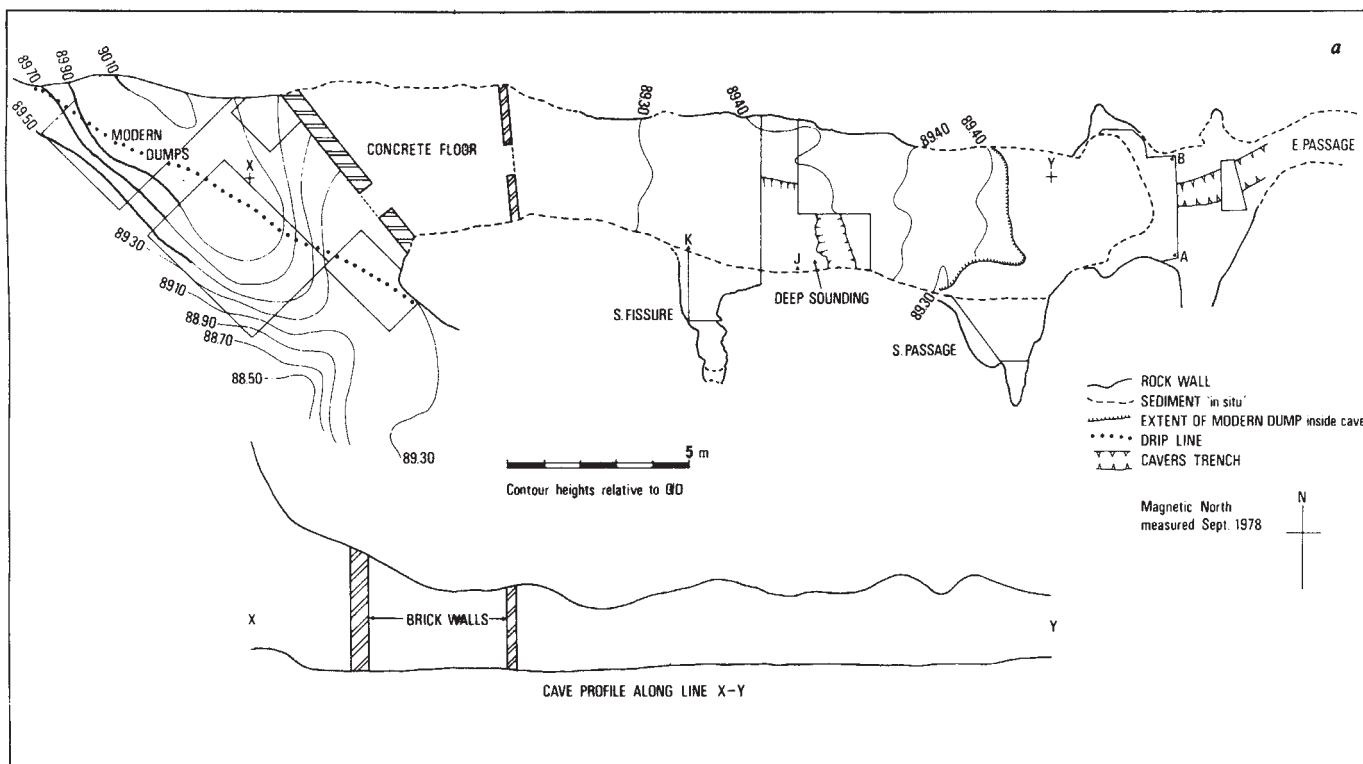
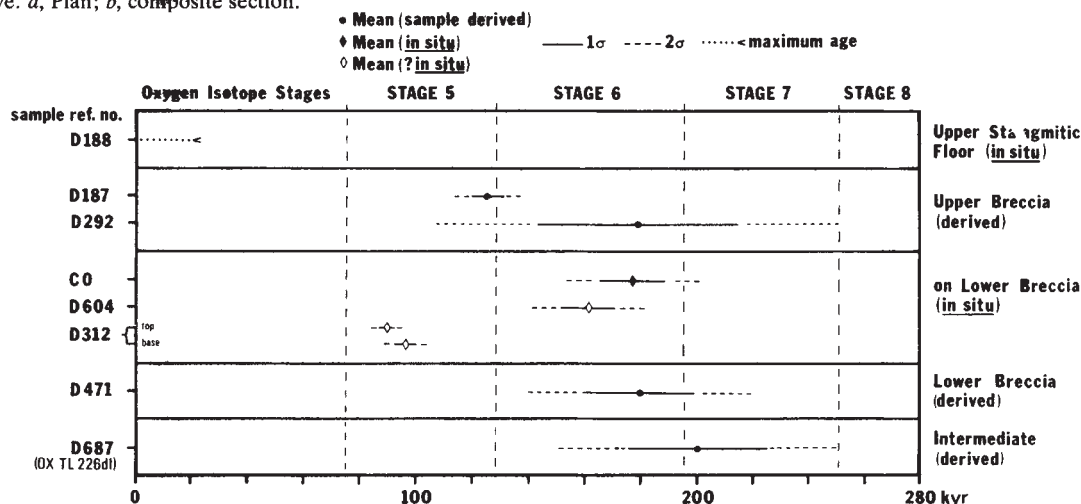


Fig. 2 Pontnewydd Cave. a, Plan; b, composite section.

Chronology

(1) Uranium series dating—The principles involved in the dating of travertines from cave-sites are described elsewhere⁵. The stratigraphical contexts of the dated samples from the South and East Passages and their locations in the cave are given in Fig. 3. Four of the samples were probably *in situ*. Of these D188 yielded a maximum age of 20 kyr, suggesting a late Devensian or Holocene age for the stalagmitic floor which caps the sequence; CO and D604 are important as, in combination, they yield a minimum age of 170 kyr for the emplacement of the Lower Breccia in which the bulk of the archaeological finds is contained. The determination of 180 ± 20 kyr (D471), on derived stalagmite from the Lower Breccia, is consistent with this minimum age. Two dates centred on 90 kyr from a small stalagmitic boss probably *in situ* on the Lower Breccia (D312) suggest a long period of quiescence before the incoming of the Upper Breccia, and a further date of 125 ± 6 kyr (D187), equivalent to Oxygen Isotope Stage 5e (ref. 6), on a stalactite incorporated into the Upper Breccia seems to confirm this and suggests that the solifluction of this layer occurred at a later cold stage. We believe that the cave had been sealed in the entrance area by the Lower Breccia deposit and so remained until this plug was breached during downcutting (through drift) of the

Fig. 3 Pontnewydd Cave uranium series and thermoluminescent dates. C, South Passage; D, East Passage.



Elwy Valley⁷, resulting in the emplacement of the Upper Breccia deposit. This view is supported by oxygen isotope analysis of single growth layers of stalagmite D312 which show it to have been deposited in equilibrium with its feed waters, a characteristic of deposits found in sealed caves⁸.

(2) Uranium relative dating—Ten bones including one modern dog femur, seven bear bones representative of the different levels in the cave, and the human mandible and vertebra were submitted for relative dating. Post-depositional uptake of uranium series elements was assessed by counting β -particle emissions⁹. All the bones, except the modern dog, gave counts higher than background, and it can be inferred that they, including the two human bones, are of some antiquity. The bones from the Upper and Lower Breccias, however, could not be distinguished, suggesting that they are not of greatly differing ages.

(3) Thermoluminescence dating—The ages obtained for emplacement of the Lower Breccia directly date neither the contained 'industry' nor the fauna beyond giving a minimum age of 177 kyr. We are fortunate in that a thermoluminescence (TL) date has been obtained from a globular burnt flint core from the Intermediate Complex immediately below the base of the Lower Breccia in the East Passage. The TL date is significant not only because it probably dates the use of fire on the site, almost certainly to be associated with the human occupation, but also because the hominid tooth described below was discovered within the same layer and in close proximity to it (Fig. 3). The date obtained is 200 ± 25 kyr (OXTL 226d1). The burnt flint core (D687) has been dated using the method previously described for flint¹⁰. TL parameters are as follows: Archaeological dose (29.6 ± 1.0) krad for $1-8 \mu\text{m}$ grains; (28.7 ± 1.0) krad for $90-150 \mu\text{m}$ grains; a value¹¹ 0.085; plateau region $375-475^\circ\text{C}$; internal dose $0.025 \text{ rad yr}^{-1}$. Soil γ + cosmic dose: $0.120 \text{ rad yr}^{-1}$ derived from fluorite dosimeter measurements in the layer. (Cosmic ray dose $0.003 \text{ rad yr}^{-1}$, on site γ spectrometer measurement.) Excavation water content 0.8 of saturation, which was 25% expressed as a percentage of dry weight. Assumed soil water content over the burial period (0.7 ± 0.3) of saturation. Subsidiary measurements for Lower Breccia, Intermediate and immediately underlying Basal Sands and Gravels indicate radioactive homogeneity. TL age (200 ± 25) kyr: lab. ref. no. OXTL 226d1. The error quoted is the total predicted error at the 68% confidence level calculated as previously described^{12,13}.

Hominid finds

Of the three hominid finds, one, a tooth (Pontnewydd 1) whose stratigraphical position is indicated in Fig. 2b, is described below. The others, an immature mandible fragment (PN 2) and a vertebra (PN 3), are both from unstratified contexts but uranium relative dating indicates no difference in age between these specimens and the remainder of the Pleistocene fauna.

The human tooth from Pontnewydd is a left upper molar, probably an M^2 of an adult individual. It has a large and low

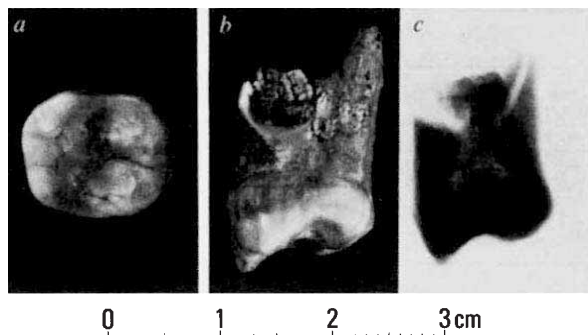


Fig. 4 The human molar: a, occlusal view (distal side at bottom); b, distal view; and c, radiograph of distal view.

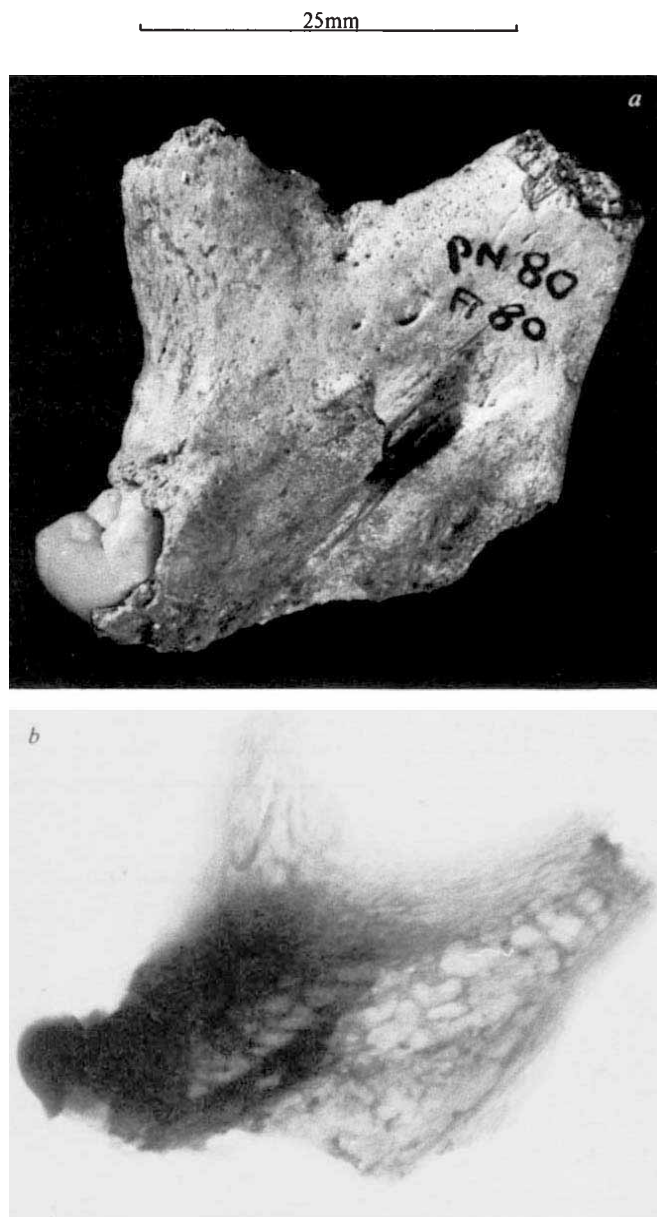


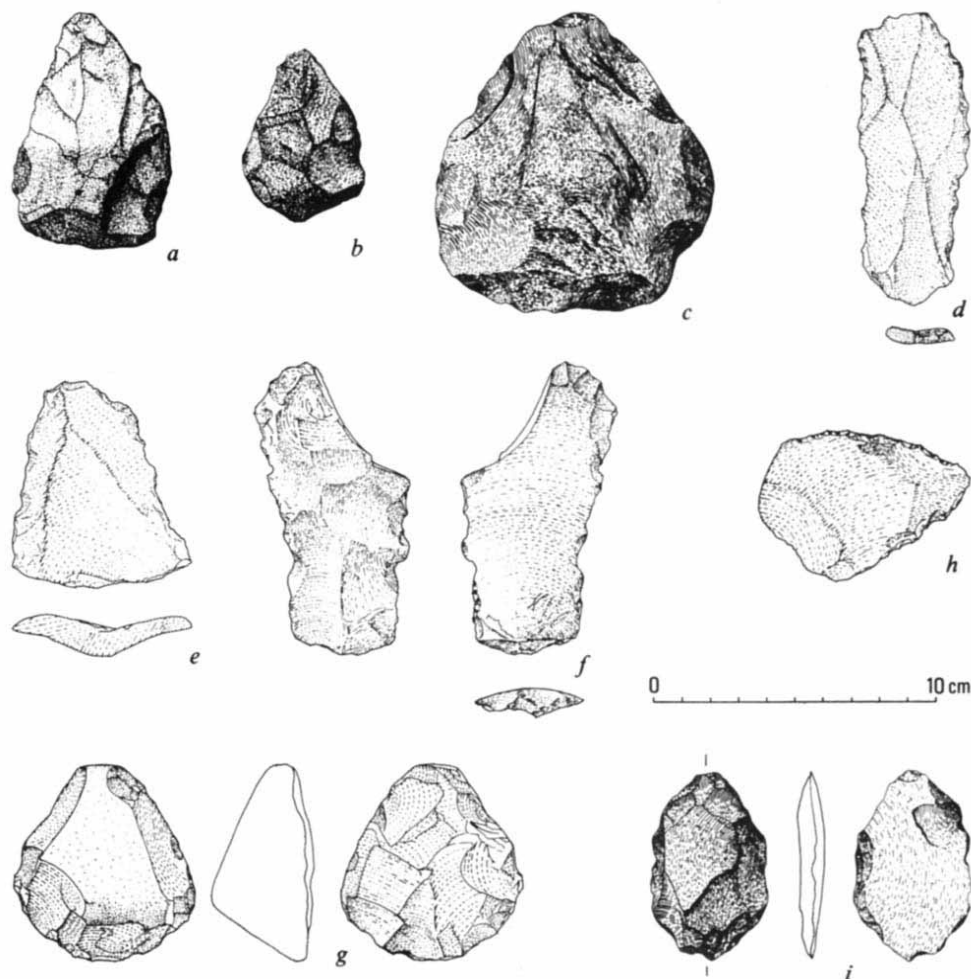
Fig. 5 The mandibular fragment: a, medial view; b, medial radiograph.

rectangular crown and a complete lingual root, but the buccal roots are broken (Fig. 4a, b).

The molar has four cusps. The protocone is the largest, the paracone is slightly larger and higher than the metacone, while the hypocone is small. The buccal cusps are higher than the lingual cusps and initial dentine exposure is apparent on the protocone. An oblique crest joins the protocone and metacone, and there are only moderately marked buccal and distal grooves. The lingual crown surface is more convex than the buccal surface, but there is no Carabelli's pit or cusp on the lingual surface, nor is a cingulum present. There are clear interstitial contact facets on both mesial and distal surfaces. The buccal roots are still fused at the point where they are broken, and taurodontism (enlargement of the pulp cavity) is associated with coalescence of the roots over half the length of the lingual root¹⁴. When intact the roots apparently barely diverged from each other, but they show a slight distal inclination.

Radiographs (Fig. 4c) of the molar show marked enlargement of the pulp cavity into the roots, which assume a prismatic shape. The form of the pulp cavity corresponds to Kallay's category of 'radicular endotaurodontism'¹⁵, where the cavity displays an hour-glass shape. This form of taurodontism is present in ~40% of permanent molars examined from the last interglacial-early

Fig. 6 Pontnewydd Cave. Artefacts: *a-c*, handaxes; *d-f*, Levallois débitage; *g*, unstruck Levallois core; *h, i*, scrapers.



last glaciation site of Krapina, Yugoslavia^{15,16}. The Krapina sample displays the highest reported frequency and degree of such taurodontism, but taurodontism is not confined to Neanderthal fossils, nor is it characteristic of them in general. Comparative data on the distribution of this character are lacking for many middle Pleistocene hominids, although the Choukoutien and Mauer dentitions show a moderate degree of taurodontism¹⁷. On the other hand, the Bourgeois-Delaunay molars, stratified both in and immediately below a stalagmitic floor attributable to a temperature phase on pollen analytical grounds but dated by the U-Th method to 146 ± 16 kyr, are reported to show none^{18,19}. Therefore the Pontnewydd molar may represent one of the earliest known examples of this degree of taurodontism in the fossil hominid record.

Table 1 lists measurements for the Pontnewydd molar and a comparative set of measurements on the M^2 of a *Homo erectus* sample, and of selected European middle and upper Pleistocene hominids. The crown dimensions and crude crown area of the Pontnewydd molar are close to the mean values of the *Homo erectus* and Krapina Neanderthal samples. The crown dimensions of the tooth also fall well within the size range of a last glaciation European Neanderthal sample, and within the upper limit of the range of an early Upper Palaeolithic sample. However, the molar is smaller than those of the middle Pleistocene sample (Petriona 1 (ref. 20), Arago 21 and the Bilzingsleben M^1 or M^2 (ref. 21)), while probably larger than that of the Steinheim specimen (comparative data on original not available). The Pontnewydd molar is identical in breadth to the mean value of a last glaciation Neanderthal sample, but is longer than most such specimens. Data on root dimensions are unavailable for many fossil hominids, but comparisons with Weidenreich's data (Table 1) suggest that the Pontnewydd molar has long and robust roots.

If the molar is an M^2 , then the complete root indicates an individual of at least adolescent age. Furthermore the presence of a distal interstitial wear facet suggests that M^3 was also in occlusion. However, the moderate degree of occlusal wear on the Pontnewydd molar suggests that this tooth derived from a young adult individual.

Some preliminary comments can be made about the immature mandibular fragment (PN2) and the vertebral fragment (PN3). The mandibular fragment represents part of the right ascending ramus, together with the crown of a molar, without developed roots (Fig. 5a). The ascending ramus lacks the upper parts of the coronoid and condylar processes and the lower border, and its minimum breadth is 31.5 mm. Exact identification of the molar is difficult, but it is small (mesio-distal length 11.2 mm; buccolingual breadth 10.5 mm; crude crown area 117.6 mm^2) and has a simple five-cusped pattern. From the condition of the molar and the fact that radiography (Fig. 5b) shows the absence (presumed developmental) of a more posterior molar, three possible ages for the individual could be proposed, based on modern developmental patterns, assuming the preserved molar is an (1) M_1 , (2) M_2 , (3) M_3 , respectively. The first possibility, implying an age of ≤ 3 yr, can be excluded by the size of the ascending ramus. The second possibility, implying an age of 8–9 yr, would enable the size of the ascending ramus to be matched with those of earlier upper Pleistocene hominids such as Teshik-Tash 1 and Irhoud 3, which are at broadly comparable developmental stages²². In this case the relatively large ascending ramus would favour its identification as a non-anatomically modern specimen. The third possibility would imply a developmental age of > 11 yr; the ascending ramus dimensions would then favour the identification of an anatomically modern, rather than archaic, morphology. However, whether the molar is an M_2 or M_3 , its simple crown morphology, without the

accessory cusps so often found in middle and earlier upper Pleistocene specimens, and its small size (near or below the minimum values for length and area in the large Krapina sample²³) suggest caution about identifying the mandibular fragment as Middle Pleistocene in age.

The vertebral fragment represents the posterior and inferior parts of the body of a middle thoracic vertebra, probably from an adult individual. Two costal facets are preserved on the inferior part of the body, but the fragmentary nature of the specimen and its unremarkable morphology preclude further comment.

Pleistocene mammals

Faunal remains occur in three successive lithological units, the Intermediate Complex and the Lower and Upper Breccias. The following list includes the mammals from the Lower Breccia and Intermediate Complex of the East Passage only.

Ochotona pusilla: Pika
Lepus cf. timidus: Hare
Castor fiber: Beaver
Lemmus lemmus: European lemming
Arvicola terrestris: Water vole
Microtus oeconomus: Northern vole
Microtus gregalis: Tundra vole
Apodemus sp.: Mouse
Vulpes vulpes: Fox
Ursus sp.: Bear
Panthera leo: Lion
cf. Panthera sp.: Leopard-sized cat
Equus sp.: Horse
Dicerorhinus kirchbergensis: Rhinoceros
Cervus elaphus: Red deer
Rangifer tarandus: Reindeer
Bos or *Bison* sp.: A bovid
Ovis cf. antiqua: Sheep

With the exception of *Dicerorhinus kirchbergensis*, widely regarded as an interglacial browser²⁴, these animals could be grouped into a fairly unremarkable cold climate assemblage, although most of the larger species tolerate a wide range of thermal environments. Nothing in this collection indicates a date more precise than the later part of the Pleistocene. Sutcliffe and Kowalski²⁵ have suggested that *Microtus gregalis* is confined to the Devensian Stage (Last Glaciation) in Britain, even though it is recorded from earlier deposits in adjacent parts of continental Europe. This proposal would seem unlikely. The characteristic rodent assemblage of the colder regions of the northern continents was well established in the early Pleistocene²⁶, and small mammal faunas closely parallel to that of Pontnewydd still exist over wide areas of northern Eurasia, Alaska and Arctic Canada. Successive cold stages during the Pleistocene could have led to the spread of rather similar faunas into Britain, particularly as low sea levels would allow free migration from the continental landmass. One would not expect any one form to be specifically excluded from the British region under these circumstances and the occurrence of *Microtus gregalis* from a pre-Devensian context is not surprising. Note here the first record of *Ovis cf. antiqua* in Britain.

The fragmented and abraded nature of the animal remains, together with the occurrence of robust limb bones that have been shattered and bent into bizarre shapes, support the suggestion that the Breccias have been sludged into their present position. Any original stratigraphy seems to have been completely destroyed and the fauna almost certainly has been mixed by this sludging process, but the abundance of carnivore remains, particularly those of adult and juvenile bears, implies that a genuine cave assemblage is present.

We make no comment about the occurrence of animals, noted by Boyd Dawkins² and McKenny-Hughes³, that would now be assigned to the Ipswichian s.s. ('Last Interglacial'). These include *Hippopotamus amphibius*, *Palaeoloxodon antiquus*, *Dicerorhinus hemitoechus* and *Crocota crocata*. None of these records have been substantiated by recent excavation. The evidence of stable isotope analysis that the cave may have been

closed during the Ipswichian leaves open the possibility that the animals may have come from a localized entrance deposit, since destroyed.

Archaeological material

The new archaeological finds (Fig. 5) comprise some 300 artefacts. Stratified examples were located, along with the fauna, within the breccia units and in the Intermediate Complex. No *in situ* settlement survives and all of the artefacts have been transported into the cave by mass movement. We cannot demonstrate that the finds represent a single industry but the location of the site far outside the known distribution of British Acheulian sites would render multiple occupation less likely. It is uncertain as to what part of the fauna may constitute the food bones of the Palaeolithic hunter-gatherer community (or communities).

Combination of the TL and uranium dates and faunal evidence suggests that the occupation(s) took place within the time range of Oxygen Isotope Stage 7/earlier Stage 6. This dating makes sense in climatic terms in so far as the occupation seems to have preceded the onset of glaciation later in Stage 6 when deep-sea core data²⁷ indicate an increase in ice-volume, and stable isotope studies of northern England speleothems²⁸ suggest glaciation from 170 to 140 kyr. Radiometric dating of the Barbados coral terraces²⁹ suggests a major eustatic lowering of sea level, bracketed by the Kendal Hill and Rendezvous Hill terraces between 180 and 125 kyr.

The archaeological material has been considered elsewhere³⁰. The principal artefactual components are handaxes of Acheulian types, other implements (principally scrapers) and Levallois *débitage* (including cores) (Fig. 6). The nature of the artefacts and small size of the potential living-area in the cave entrance suggest that the cave was probably used as a temporary butchering site. The industry finds little close comparison with other sites either in Britain or in adjacent continental areas.

Of the 300 artefacts recovered about 40 are of flint or chert and most of the remainder are of siliceous igneous, pyroclastic and volcanoclastic rocks. These specimens, so far only examined macroscopically, show evidence of having suffered a low-grade of metamorphic recrystallization, whilst in addition a cleavage is variably developed within the rocks, being most pronounced in the fine-grained specimens.

The variety of rock types, the low grade metamorphic crystallization and the poorly developed cleavage, suggest a source either in North Wales or the English Lake District. Their presence on the site, whether as artefacts in the Breccias or as unmodified pebbles in both the Breccias and Basal Sands and Gravels, suggests glacial transport to the Elwy Valley before oxygen isotope Stage 6. Whatever the origins of the other rocks, the flint (which has been recognized so far only from the Breccias) must have come as a component of the Irish Sea drift³¹. This drift may be compared with the 'Main Irish Sea Glaciation', of Embleton³² whose hypothesis of the cutting of the lower Elwy Valley as a result of local glacial diversion of the river by inland penetration of Irish Sea Ice would then presuppose another such event earlier in the glacial Pleistocene, unrecognized as yet in the local terrestrial till sequences.

The Pontnewydd excavations will continue for several more seasons to increase the statistical validity of the artefactual and other samples; to improve the dating and comprehension of the stratigraphy; to establish the relationship of the site to the local Quaternary geology and geomorphology; and, if possible, to recover more hominid remains.

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and its principal inspector, Dr M. W. Thompson. The finds are lodged in the National Museum of Wales by gift of the landowner, Major David Williams-Wynn whose interest and support has made these discoveries possible.

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Intragenic amplification and divergence in the mouse α -fetoprotein gene

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The DNA sequences of the 14 exon junctions in the murine α -fetoprotein gene were determined using cloned genomic DNA. When these exons were examined with respect to the polypeptide segments they encoded, a direct correspondence between a threefold repeat of four exons and three protein domains was observed. Nucleotide sequence comparisons among the four exons of each domain were used to deduce the likely structure of the primordial domain, and the order and mechanism of its triplication to form the tripartite ancestral gene from which both α -fetoprotein and serum albumin arose. Sequence homologies among the four exons that constitute a single domain also suggest that they were derived, at least in part, from a common sequence which underwent successive amplification and divergence.

MAMMALIAN α -fetoprotein (AFP) and serum albumin are useful models for elucidating early events in the evolution of a multi-gene family. Their genes arose through duplication of an ancestral gene 300–500 Myr ago^{1–6}. They have remained linked on chromosome 5 in the mouse, with the albumin gene 14 kilobases (kb) of DNA upstream from the AFP gene^{7,8}.

Brown^{9,10} first suggested that the ancestral gene itself had been formed by amplification and divergence of a simpler sequence when he recognized a thrice-repeated pattern of cysteine–cysteine disulphide bridges in the amino acid sequences of human and bovine albumins. He concluded that the albumin gene must have arisen from the triplication of a primordial domain, consisting of ~190 amino acids. The amino acid sequence of murine AFP also shows this threefold repeat pattern^{1,6}.

We have inquired into the genetic basis for the tripartite structure of the AFP/albumin ancestor. An examination of the structures of their genes in the mouse² revealed that both are comprised of 15 coding blocks or exons, which are interrupted by 14 intervening sequences. The sizes of the 15 corresponding exons in each gene were estimated by electron microscopy of R-loops, and shown to be very similar. Of particular interest was the observation that the 12 internal exons of either gene, numbers 3–14, seemed to be composed of three similar sets of four exons, based on their sizes. We therefore proposed that the three protein domains had been generated by the triplication of a primordial gene consisting of four exons². To test this, the borders of the 15 exons of the AFP gene were located by DNA sequencing using cloned genomic DNA.

Nucleotide sequences of the splicing junctions of the AFP gene

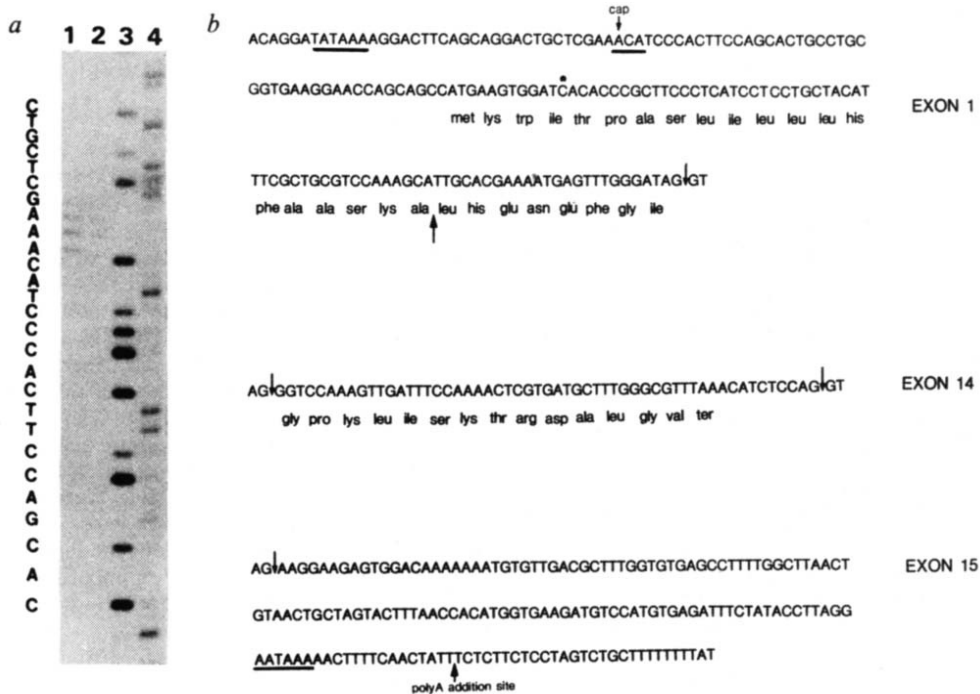
The borders of the exons in the mouse AFP gene (see Fig. 1) were determined by the procedure of Maxam and Gilbert¹¹. In some instances where the determination of a splicing site was unequivocal from sequences of one side of the intron, the sequence at the other side was not determined. The intron junction sequences closely resemble the consensus sequence suggested by Lerner *et al.*¹², and no exceptions to the GT–AG rule proposed by Breathnach *et al.* were seen¹³. Indeed, because of the presence of sequence redundancy at 12 of 14 borders, the exact locations of the splice sites can only be assigned on the basis of these four invariant nucleotides.

The location of the mRNA cap site was determined by an S₁ nuclease protection experiment¹⁴ shown in Fig. 2a. A 100-nucleotide DNA fragment, labelled on the anticoding strand at a Sau3A site in exon 1, was hybridized to AFP mRNA. The S₁ nuclease-resistant fragments (lane 1) were sized on an acrylamide gel by comparison with chemical degradation products of the same DNA fragment (lanes 3, 4). Three prominent bands, 42–44 nucleotides upstream from the AUG initiation codon, were observed. From a comparison of the nucleotide sequence at the putative cap site with previously reported cap site sequences in other genes^{15,16}, we have tentatively identified the first adenine residue of the underlined ACA triplet in Fig. 2b as the cap site. The other bands presumably reflect imprecise cutting by S₁ nuclease as they did not decrease with increasing S₁ nuclease concentration (lane 2). A 'TATAA' sequence, or

	EXON #	EXON SIZE (bp)	3' EXON JUNCTION	5'	IVS	3'	5' EXON JUNCTION	EXON #
	1.	129	66G ATA G		GTAGAT ... TTTTCAG		CT TCC ACG	2.
	2.	52	CTT AGC AT		GTAGTT ... CTGTCAG		A GCT ACC	3.
I	A 3.	121	GAA AGC CAG		GTAGTG ...		CTA TCT GTG	4.
	B 4.	212	ATG AAC AG		GTAGGA ...		G TTC ATC	5.
	C 5.	133	CAG ACA AAG		... CATCTAG		AGA GCA TCC	6.
	D 6.	98	CAG GCA AC		GTAGTA ...		A ACC ATT	7.
II	A 7.	130	CAG GAT GGG		GTAGGAG ...		GAA AAA GTC	8.
	B 8.	215	ATG GCA AG		... ATTTAAG		C TTT CTT	9.
	C 9.	133	GAC AAT CTG		GTAGTT ...		GAA GAA GAA	10.
	D 10.	98	CAA AAT CT		... CTTCAG		G TTC CTT	11.
III	A 11.	139	GAG GGA ATG		GTAGTG ... TTAACAG		GCC GAC ATT	12.
	B 12.	224	AAA CAA GA		... ACCCCAG		G CTT CTC	13.
	C 13.	133	ACA GAA GAG		GTGCGTA ...		GGT CCA AAG	14.
	D 14.	55	TCTCCAG		GTAGAA ... ATTCCAG		AAGGAAGA	15.
	15.	141						
			CONSENSUS: AG GTAGT ^Δ ... TTCCAG					

Fig. 1 The sequences at the splice junctions in the AFP gene. The partial DNA sequences spanning the 14 splice junctions of the murine AFP gene were determined using cloned genomic fragments² and the procedures of Maxam and Gilbert¹¹. The tentative locations of the 15 exons were originally determined by R-loop techniques and a direct comparison of a detailed endonuclease restriction map of the cloned genomic fragments with the restriction map of its cDNA^{1,2,46}. The positions of the junctions, indicated by the vertical lines and the sizes of the 15 exons, shown in the first column, were deduced by comparing the genomic and cDNA sequences¹ and by the presence of the invariant GT and AG dinucleotides (underlined) at the 5'- and 3'-intervening sequence borders, respectively. The sequences of the last two or three complete in-frame exon triplet codons at the 3' junction are followed in each instance by the first seven nucleotides of the adjacent intervening sequence. The last seven nucleotides of that intervening sequence and the first two or three complete triplet codons of the next exon follow the dotted line which represents those remaining intervening sequences. The in-frame triplet codons are designated by the spacing of the exon sequences. The consensus sequence of Lerner *et al.*¹² for the splice junctions is shown at the bottom. The designations used in the text for domains (I, II and III) and subdomains (A-D) in the gene, are shown on the far left.

Fig. 2 The sequence at the 5' and 3' termini of the AFP gene. *a*, The approximate location of the first nucleotide of AFP mRNA was determined by an S₁ protection experiment¹⁴. One microgramme of fetal yolk sac poly(A)⁺ mRNA, which is 10–20% AFP mRNA, was hybridized to a denatured 100-bp *Sau*3A–*Hinc*II fragment derived from pAFP14Z (ref. 2), which was end-labelled at the *Sau*3A site within exon 1 on the anticoding strand (asterisk in *b*, exon 1). The hybridization and S₁ nuclease digestion were performed according to the procedures of Favaloro *et al.*⁴⁷. The protected DNA fragment was electrophoresed in a 10% urea acrylamide gel. Lanes 1, 2: S₁ nuclease (Miles) used at 200 and 500 U ml⁻¹, respectively. Lanes 3, 4: To size the S₁ digest gel migration, the same *Sau*3A–*Hinc*II fragment was subjected to chemical degradation¹¹ for the G reaction (lane 3) and A reaction (lane 4). The complement of the determined sequence is shown on the left, so as to correspond to the coding strand. The sizes of the S₁-protected fragments were determined by comparison with the nucleotide sequence, taking into account the fact that the chemically degraded fragments lack the modified base. *b*, The nucleotide and amino acid sequences of the first and last two exons. In exon 1, the underlined ACA trinucleotide identifies the likely position of the cap sequence, as determined in *a*. The underlined TATAA sequence 30 bp upstream from the cap site is the Hogness box. The 3'-splice junction is shown by a downward arrow and the junction between the 20-amino acid signal peptide and the mature protein is designated by the upward arrow. The *Sau*3A site labelled in *a* is shown by the asterisk. In exon 14, the splice junctions are designated by the downward arrows and the terminator codon TAA is shown as 'ter'. In exon 15, the 5'-splice junction is shown by the downward arrow. The signal for poly(A) addition, AATAAA, is underlined and the upward arrow designates the poly(A) addition site⁶.



Hogness box, thought to be required for precise initiation by RNA polymerase II, is found 30 base pairs (bp) upstream from the cap site (Fig. 2*b*).

The termination codon for AFP synthesis, TAA, occurs in exon 14, as shown in Fig. 2*b*. The 3'-untranslated sequence in this exon differs at three positions from our previous report¹. Although we cannot eliminate sequencing errors as an explanation, it is likely that these result from the fact that the cDNA and genomic clones were constructed from different inbred strains. The sequence in Fig. 2 agrees with that of Law and Dugaiczky⁶. Exon 15, the last exon of the AFP gene, contains only 3'-untranslated sequence. The poly(A) addition signal, AATAAA^{17,18}, is 120 bp from the start of this exon (Fig. 2*b*) and the poly(A) addition site is an additional 15 bp downstream⁶.

Coincidence of genetic and functional domains

An examination of the sizes of the internal 12 exons in the AFP gene (Fig. 1) strengthens the original premise of a thrice-repeated pattern. The gene can be readily divided into three sets of four exons designated subdomains A–D based on their sizes; this excludes the first two and the last exons. Subdomains vary in size within families by multiples of 3 bp, and thus any insertions or deletions within subdomains do not change the reading frame of downstream exons. There is also conservation of the location within a triplet codon of the 5' and 3' borders of each family (Fig. 1).

Figure 3 shows the pattern that emerges when the borders delineating the exons are superimposed on the protein. It is immediately apparent that all the exons of any one family encode segments of the protein that are identical with respect to the location of the disulphide bridges. For example, each subdomain C (closed circles, Fig. 3) encodes an equivalent double loop region or subdomain in each of the three domains.

Are the three genetic domains defined by the four-exon repeat coincident with functional domains in either AFP or albumin? One can consider the first and last exons, which lie outside the domains, as functionally distinct in that the first exon encodes the signal peptide and the last exon encodes the

3'-untranslated sequence and poly(A) addition signal. In many but not all secreted proteins, the signal peptide exon is physically separated from the rest of the gene.

The active binding sites in mammalian AFPs are poorly characterized. In the case of albumin, experiments on the intact protein and on isolated fragments corresponding to domains have localized binding sites to specific domains. The high-affinity long-chain fatty acid binding site is in domain III, the bilirubin binding site in domain II and the indole binding sites in domain I¹⁹. Domains I and II exhibit weaker fatty acid binding sites, suggesting that these are remnants of the high-affinity site in domain III, the putative primordial domain (see below). Interestingly, copper(II) or nickel(II) binding is performed by the first three amino acids of the mature protein, which is separately encoded by exon 1¹⁹. These studies are consistent with the notion that the protein domains are functionally distinct and that the selective pressure to maintain amplified domains lay in the generation of functional diversity within the protein.

The relationship between genetic and functional domains has been clearly demonstrated only in instances where there has been intragenic amplification and divergence, as has occurred in the AFP/albumin ancestral gene. The other compelling example occurs in the immunoglobulin heavy-chain constant region genes, where each of the four constant region gene domains has been correlated with structural or functional protein domains²⁰⁻²⁵. Stein *et al.*²⁶ have reported a triplication of two internal exons in the chicken ovomucoid gene which possibly correspond to the three binding sites for protease inhibitor in the protein.

Amplification of genetic domains is facilitated by the presence of pre-existing intervening sequences; thus, as suggested by Gilbert²⁷ and Darnell²⁸, intervening sequences serve to increase the probability of useful recombination during unequal crossing-over. Following amplification, the presence of intervening sequences then decreases the likelihood of genetic recombination and subsequent deletion of DNA, because the intervening sequences themselves rapidly diverge²⁹. In the case of the albumin and AFP genes in the mouse, which are located⁸

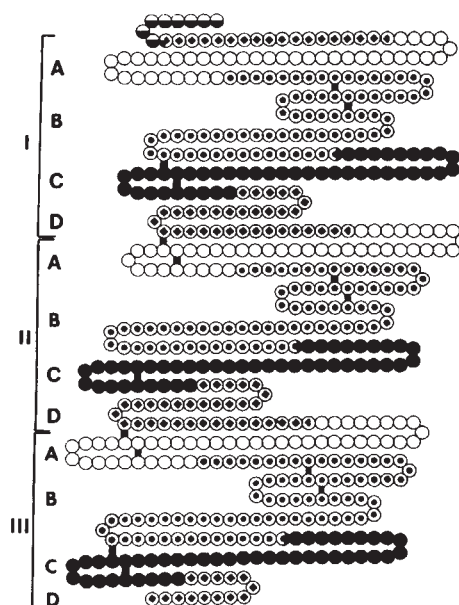


Fig. 3 The coincidence of genetic and protein domains. The 585 circles in the diagram represent the amino acids of mature AFP, drawn to connect adjacent cysteine-cysteine disulphide bridges, shown by the connecting bars^{1,10}. The amino acids encoded by exon 1 (●), the three A subdomains (○), the three B subdomains (◐), the three C subdomains (◑) and the three D subdomains (◒), are drawn, and designated on the left of the diagram according to usage in Fig. 1 and the text. In cases where a splice junction interrupts an in-frame triplet codon, the amino acid is drawn with two symbols.

13.5 kb apart on chromosome 5, this has probably contributed to the maintenance of the two genes in tandem.

When one examines genes such as insulin^{30,31}, lysozyme³² and alcohol dehydrogenase³³, in which there is no apparent intragenic amplification, the coincidence of genetic and functional domains is far less evident. Gilbert²⁷ has proposed that the selective advantage of intervening sequences lay in the ability of a cell to recruit by translocation and recombination discrete genetic domains for the assembly of new genes, thereby markedly increasing the rate of evolution. One test of such a model would be the demonstration that genes are composed of genetic elements or exons encoding specific functions of the protein. Structural data, and the observation that the haem binding site of α or β globin is encoded by the middle exon of the three-exon gene, have been cited by several groups as support for this model^{31,32,34-36}. Particularly intriguing is the finding that this exon is in fact split in leghaemoglobin of soybean³⁷, as was predicted by Go³⁶ on the basis of structural arguments alone.

Mechanisms of divergence between domains

A comparison of related subdomains provides an opportunity for identifying evolutionary mechanisms that have generated domain diversity. To facilitate such a comparison, each subdomain family was aligned using the optimal nucleotide sequence homologies found by the computer programs of Staden³⁸ and an adaptation of the Queen and Korn³⁹ program. The minimum number of in-frame gaps have been introduced to maintain lengths of uninterrupted homology.

Figure 4a shows the alignments for each subdomain family, and Table 1 enumerates the nucleotide homologies within and between subdomain families, together with the probabilities that the number of matches could have arisen by chance. The three C subdomains are identical in size, and appear to have incurred no insertions or deletions. They have been aligned in Fig. 4a with a two-codon gap to facilitate their comparison with the A family, discussed below. They are also the most closely homologous family, with a 47% nucleotide homology between IC and IIC, a 44% match between IC and IIIC and a 36% match

Table 1 Sequence homologies among subdomains

a, Sequence homology within subdomain families			
	I vs II	II vs III	I vs III
A	36.4% 1.2×10^{-2}	41.5% 6.8×10^{-6}	34.7% 6.9×10^{-3}
B	38.8% 2.7×10^{-6}	35.8% 1.3×10^{-4}	32.6% 5.9×10^{-3}
C	46.6% 2.9×10^{-7}	36.1% 1.7×10^{-3}	43.6% 3.7×10^{-7}
D	32.7% 4.0×10^{-2}	NS	NS
Total	39.1% 1.3×10^{-12}	36.9% 1.5×10^{-10}	35.4% 2.5×10^{-8}
b, Sequence homology between subdomains A and C			
	IC	IIC	IIIC
IA	NS	32.2% 3.4×10^{-2}	44.6% 3.7×10^{-7}
IIA	40.0% 3.9×10^{-5}	39.2% 1.1×10^{-4}	40.8% 1.7×10^{-5}
IIIA	40.6% 1.7×10^{-5}	41.4% 6.8×10^{-6}	46.6% 2.9×10^{-7}

a, Nucleotide homologies of pairwise comparisons within subdomain families for the alignments illustrated in Fig. 4a are shown. In each case, the upper value represents the per cent homology, codon gaps not being taken into consideration. The bottom value is the probability (P) of finding this homology or more by chance, taking the length of the sequences being compared into account. It is based on the normal approximation to the binomial distribution, using the formula

$$p = 1 - \Phi\left(\frac{H - np}{\sqrt{npq}}\right)$$

where n is the standard length of the sequence, H is the number of homologous bases, p is the probability of finding a match at any position (in this case $p = 0.25$), and $q = 1 - p$. (NS = not significant.) b, Nucleotide homologies of pairwise comparisons between subdomains A and C are shown, using the alignments illustrated in Fig. 4a.



Fig. 4 The alignment of sequence homologies within subdomain families. *a*, The nucleotide sequences of each of the 13 internal exons are aligned in their corresponding subdomain families. Their alignments were determined by optimizing nucleotide sequence homologies in pairwise sequence comparisons within families. Within each family, homologies between domains I and II and domains II and III are indicated by the vertical lines, and the homologies between domains I and III are shown as asterisks below domain III. The sequences are displayed with spaces between in-frame codons, so that codon gaps in subdomains are evident. The two-codon gap in the C family alignment facilitates the comparison of the A and C families. *b*, The bottom alignment shows a comparison between representatives of each subdomain family, IIIA, IIIC, IID and IB. The entirety of IIIA and IIIC are aligned, and the vertical lines between them identify homologous nucleotides. The first 57 bp of IID are compared with internal regions of IIIA, IIIC and the last 50 bp of IB. The homologies between IID and each of these are shown on the right. The probabilities that these could have arisen by chance, using the formula in Table 1, are 1.64×10^{-4} (IID vs IIIA), 5×10^{-5} (IID vs IIIC) and 1.3×10^{-6} (IID vs IB).

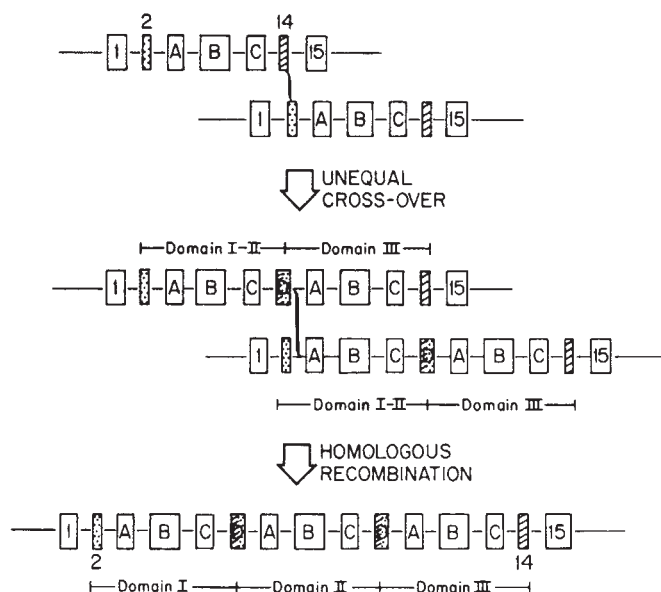


Fig. 5 A model to explain the triplication of the primordial domain. A model for the generation of the three domains in the ancestral AFP/albumin gene is illustrated. The seven exons comprising the entire primordial gene consisted of the present exon 1 containing the signal sequence, exon 2, subdomains A, B and C, exon 14 containing the termination codon, and exon 15. An unequal cross-over event between exons 2 and 14 resulted in the formation of a D exon. This created a two-domain gene, equivalent to domains I-II and III. A subsequent homologous recombination event then gave rise to domains I and II, as the result of a duplication of domain I-II. This model takes into account the sequence relatedness of the three domains in Table 1, as well as the chimaeric nature of the D subfamily.

between IIC and IIIC. The A subdomains each differ in size, and their homologies range from 35 to 42%, the longest, IIIA, being 139 bp long, 9 bp longer than IIA. A single gap in IIA, 30 bp from the 3' border, represents the most likely position of either a deletion in IIA or an insertion in IIIA. Subdomain IA contains an additional gap of three codons, which has been positioned to the 5' side of the gap in IIA. In this case, however, there are several other possible locations, and the alignment of the four codons to the right of the gap is tentative.

By comparing the A subdomains with their counterparts in the rat albumin gene, whose sequences were determined by Sargent *et al.*^{40,41}, it is possible to date approximately when these gaps occurred. Although subdomains IIA and IIIA are identical in size in both genes, albumin IA is four amino acids longer than AFP IA. Therefore, one would infer that the size difference between IIA and IIIA existed before the AFP/albumin gene duplication, tentatively placed at 300–500 Myr ago², whereas the alteration in IA occurred after it.

Alignment of the B subdomain family requires that two members contain deletions, at different places in the sequence. As illustrated in Fig. 4, IB (212 nucleotides) contains two gaps, one of 9 bp and one of 3 bp. IIB (215 bp) contains a gap of one codon at its 5' border, which is the only possible example of a mutation at a splice junction, and a second codon gap elsewhere. The alterations in the B subdomains are apparently unrelated, and probably arose independently after the domain triplication by deletions, for otherwise one must postulate two insertions at each of the gaps. This is in keeping with the finding by de Jong and Ryden⁴² that deletions are far more common than insertions as evolutionary mechanisms of divergence. The members of the B family are less homologous to one another than are those of the A or C families, with pairwise homologies of 32–39%.

The D family of subdomains consists of four exons, exons 6 (ID), 10 (IID), 14 (IIID) and 2. This classification is based on several different lines of argument, as the total percentage homology between members of this family is less than in any of the other families (see Table 1). Exons ID and IID are identical in size, with the corresponding cysteine residue occurring at the same position, but only share marginal nucleotide homology. This is not surprising, given that of all the exons in the AFP gene, that encoding ID is the least homologous to the corresponding exon in the rat albumin gene⁴¹. Thus, we consider that there is little selective pressure to retain the constancy of the nucleotide sequence of ID, although there is obviously considerable pressure to maintain the size of this subdomain at 98 bp. The relationship between IID and IIID is most evident when each is compared with an internal region of either IIIA or IIIC. As shown for IID in Fig. 4b, its first 57 bp are 46 and 47% homologous to a 57-bp internal segment of IIIA and IIIC, respectively. Similarly, IIID is 45% homologous to the same region in either IIIA or IIIC (data not shown). Finally, the inclusion of the second exon in this family, which had previously been considered outside the domain repeats, is based on a 40% homology between it and the final 52 bp of IID, as shown by the asterisks below IID in Fig. 4a.

Internal homologies shared among subdomain families

An examination of the evolution of the primordial domain itself was initiated when marked similarities between the A and C subdomain families became apparent. They are very similar in size and in the pattern by which the exon junctions occur in the reading frame. In fact, it is possible to align them as a six-member family (see Fig. 4a), based on significant nucleotide homologies which they share (Table 1). As shown in Fig. 4b and Table 1, subdomains IIIA and IIIC are as homologous to each other, 47%, as the most homologous pair within either family, IC and IIC. Thus, subdomains A and C must have arisen from a duplication which preceded the formation of the primordial domain.

Several conclusions about the history of the A/C family can be drawn by comparing the six related subdomains. It is likely that the original A/C exon was 133 bp long, and that the additional 6 bp in IIIA resulted from an insertion (Fig. 4b). Otherwise at least two exons would have had to undergo independent but identically positioned deletions before their further amplification. For the same reason, the gaps present in IA and IIA are the likely consequence of deletions. It has been proposed, based on sequence analysis of globin genes in mammals, that deletions and insertions occur preferentially in regions composed of short direct repeats¹⁵. Evidence of such an event is seen when the 3'-untranslated sequence of the mouse albumin gene is compared with that of the rat⁸. If such a sequence was present in the primordial A/C subdomain, this could have allowed independent deletions at the same location. However, no such repeated sequence in IIIA is apparent today.

We next investigated whether sequences existed in the other two subdomain families, B and D, which would suggest a common ancestry with A and C. The results indicate that all four subdomain families exhibit regions of homology with one another. In particular, as aligned in Fig. 4b, the first 57 bp of subdomain IID are 54% homologous with the last 50 bp of IB. This same region of IID is homologous with the middle of IIIA and IIIC. A more detailed analysis of these inter-subdomain relationships is now under way, but it is already evident that all four exons that constitute a domain are related, and were probably initially derived, at least in part, from one exon.

The sequence of events leading from a single exon to the primordial domain, composed of exons of quite different sizes, must have included amplification of that exon (of as yet undetermined length) creating multiple adjacent copies separated by intervening sequences. Following their divergence, separate exons could have been consolidated by unequal cross-

overs during recombination or by removal of intervening sequences, similar to that which has occurred in a rat insulin gene³⁰ and in a pseudo α -globin gene in the mouse⁴³. Such a sequence of events has been postulated for the type 1 α 2 collagen gene of the chick, which contains multiple homologous 54-bp exons⁴⁴. Mutations within junction sequences could also result in exon border changes, altering the size of individual exons and adding segments unrelated in sequence to the initial exon. Such processes would eventually have led to the establishment of a primordial gene, the exons of which varied in size, but which were at least partially related in sequence.

Brown^{9,10} and McLachlan and Walker⁴⁵ have presented a model to explain the generation of the primordial domain, based on the positions of the cysteine-cysteine bridges within a domain. Their model proposed that a 73-amino acid segment triplicated, and then a middle segment was consolidated by deletion. In genetic terms, their 73-amino acid segment would have been encoded by exons 2, A and 14, and the triplication would have generated a gene composed of 9 exons flanked by exons 1 and 15. The subsequent generation of exon B from the internal exons 14, 2, A, 14 and 2 must have involved deletions of both coding sequence and intervening sequences, leaving a primordial domain composed of exons 2, A, B, A (C) and 14. The homologies we observe between exon families certainly support a mechanism of this kind although the precise details of the deletions have not been elucidated. Note that the homologies between exons A and 14 also argue that intragenic amplification must have occurred before the establishment of the 73-amino acid segment.

Order of domain triplication

Pairwise comparisons between the nucleotide sequences of the three domains can potentially be used to trace the order of the two successive duplications of the primordial domain. The sequence homologies between domains reflect, in part, the evolutionary time between successive domain duplications, the most homologous corresponding to the most recent duplication. However, it should be emphasized that other evolutionary pressures exerted at both the nucleotide and amino acid levels have contributed to the rate of divergence of the three domains, and may serve to obscure the divergence based on time.

Although comparison of the homologies between entire domains (Table 1) shows domains I and II (39%) to be more closely related than domains I and III (35%), this difference is not statistically significant by a χ^2 test. Using this test, the only significant difference between the homologies within any subdomain family occurs in the C family, where the relatedness of domains I and II is significantly greater than that of II and III. Nevertheless, it is also evident that in three of the four families (B, C and D), domains I and II are the most homologous, and in subdomains A, B and D domains I and III are most distantly related. It seems likely, therefore, that domain II is the evolutionary link between domains I and III, and the order of triplication was domain III giving rise to a domain which subsequently duplicated to form domains I and II. A brief examination of rat albumin sequence⁴⁰ reveals that the overall order of relatedness between domains is the same as that in AFP.

This order, which was originally predicted by Brown¹⁰ based solely on amino acid homologies in bovine and human albumins, is supported by a model to explain the generation of the D subfamily. As discussed earlier, and represented in Fig. 4, this family is composed of two 98-bp exons, ID and IID, which are homologous at their 5' termini to exon 14 (IIID), and at their 3' termini to exon 2. Possibly, at one time exons 2 and 14 were each entire subdomain Ds, and in two independent events the beginning of exon 2 and the end of exon 14 were deleted. Alternatively, one could postulate a copy of D split, creating two separate exons. If an exon division did occur, it is difficult to envision how exons 2 and 14 came to lie on opposite ends of the gene.

We favour a model in which exons 2 and 14 flanked the primordial domain, corresponding to the A, B and C subdomains of domain III (Fig. 5). During the first unequal cross-over in the triplication, these flanking exons fused to form subdomain D. This could occur in one step, with the cross-over occurring within the two exons, eliminating the termination codon and generating a two-domain protein. Alternatively, it could occur in two steps, the first step being a cross-over in the intervening sequences, followed by a fusion of exons 14 and 2. In the latter case, however, the resulting protein would still only comprise a single domain, until subsequent events removed the termination codon in the fused subdomain. This second alternative seems unlikely, as it would place the downstream domain under no selective pressure. In either case, the one strong constraint placed on the fusion would be that the reading frame of the downstream exons be preserved. The subsequent duplication of the left domain, by homologous unequal crossing-over, would generate domains I and II, in agreement with the overall relatedness of domains I and II over domain III.

The model in Fig. 5 differs slightly from our original proposal²

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in that the primordial genetic domain is composed of five rather than four exons, and exon D is only generated by the fusion of two of these during or after the first duplication. Thus, one could define a domain as consisting of the equivalents of exons 2, A, B, C and the first half of exon D. This is precisely the way in which the three protein domains in albumin were originally delineated^{10,45}. The second duplication of exons I-IIA, B, C and D is in keeping with our original model.

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Membrane asymmetry in epithelia: is the tight junction a barrier to diffusion in the plasma membrane?

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Membrane-bound lectins and some lipid probes are incapable of passing through the tight junction region of epithelial cell membranes. The lectins are immobile on the cell surface, but lipid probes diffuse freely in the membrane. The ability of a lipid probe to pass the tight junction is correlated with its ability to 'flip-flop' to the inner monolayer of the cell membrane bilayer.

EPITHELIA form tight monolayers of cells in many organs and control the passage of solutes across the monolayer. Individual cells are asymmetric, with the apical (mucosal) and basolateral (serosal) surfaces exhibiting different membrane morphology, ionic permeabilities, distribution of enzymes and sensitivity to hormones and drugs¹⁻⁴. This asymmetry is correlated with the

presence of an intact tight junction which encircles the cell at the boundary separating the apical and basolateral surfaces⁴⁻¹¹. The tight junction seems to form a barrier to extracellular penetration of solute and water across the monolayer⁴.

The mechanisms of development and maintenance of cell asymmetry are unknown. It has been suggested that the tight junction may act as a barrier to lateral diffusion of membrane constituents between apical and basolateral surfaces^{4,6,8,9,11}. We report here the results of experiments designed to test that

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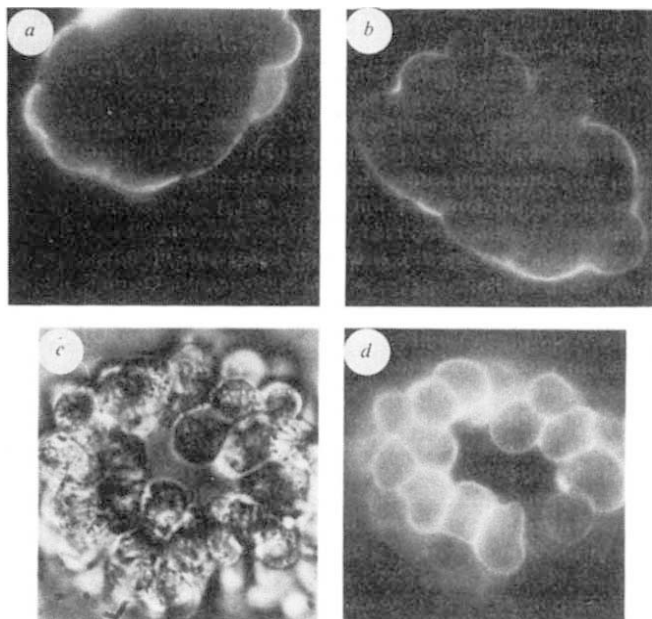


Fig. 1 Fluorescence micrographs of domes of A6 cell monolayers labelled with tetramethyl rhodamine-conjugated wheat-germ agglutinin (a), AFC₁₆ (b) and diIC₁₆ (d). c, Brightfield photograph of the dome in d. In all cases, the cells were labelled by adding the fluorescent probe to the medium bathing the apical surface of the cell monolayer. In this way, only apical membranes were labelled initially. The plane of focus is part way up a dome of cells. In this geometry, labelling of the apical surface alone appears as a series of arcs, as in a and b, and labelling of the entire plasma membrane appears as complete rings, as in d. The apparent gap in the ring of fluorescent cells in d is due to a cell which was weakly labelled with diIC₁₆, as occasionally happens with this probe. Labelling conditions were: 25 µg ml⁻¹ wheat-germ agglutinin for 20 min; 10 µg ml⁻¹ AFC₁₆ for 10 min; 5 µg ml⁻¹ diIC₁₆ for 5 min. ×600.

suggestion. We selectively labelled with a fluorescent probe one surface of a confluent monolayer of epithelial cells grown in culture, and then determined the rate at which the probe diffused laterally in the membrane and whether it could move through the region of the tight junction to the side of the cell not labelled initially (for example, from the apical to basolateral surface). We first measured lateral diffusion coefficients using the technique of fluorescence photobleaching recovery (FPR)¹², and then monitored the amount of fluorescence on the apical and basolateral surfaces to determine any movement through the tight junction. We found that cell-surface sites labelled with fluorescently conjugated lectins were unable to move through the region of the tight junction and, in fact, were completely

immobile with respect to lateral diffusion. On the other hand, fluorescent lipophilic probes diffused freely, and fell into two categories: those capable of passing the region of the tight junction by lateral diffusion in the membrane, and those incapable of penetrating the tight junction. These results show that epithelial cells are capable of segregating membrane constituents as small as lipids on one side of the tight junction, thus suggesting a mechanism whereby epithelia may maintain different lipid compositions on the apical as opposed to the basolateral membranes^{3,13}. As the ability of a lipid probe to pass through the tight junction is also correlated with its access to the inner monolayer of the cell membrane bilayer, we suggest that the tight junction presents an impassable barrier to lipid components only in the outer leaflet of the membrane.

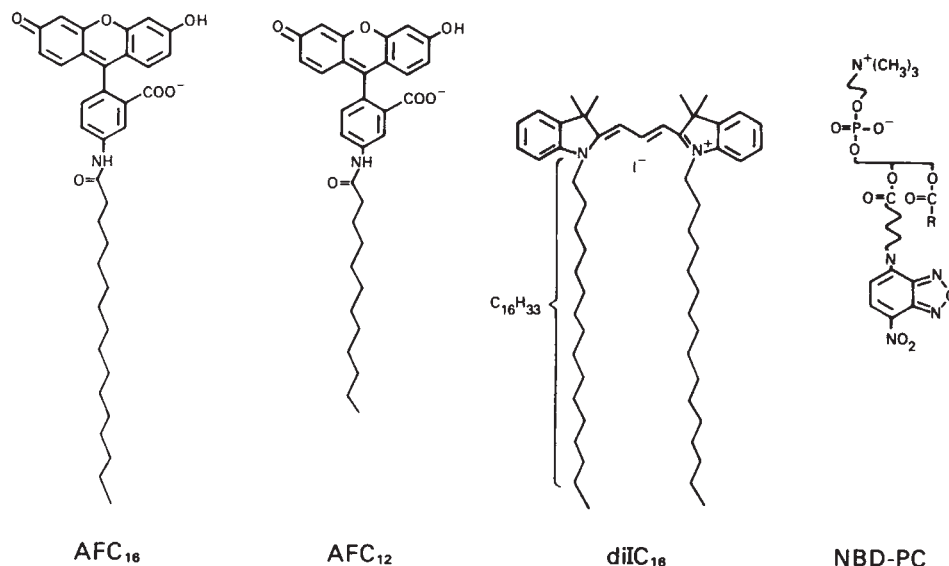
Lectin probes

Continuous epithelial cell lines were grown to confluency in culture according to methods published elsewhere^{4,14,15}. Cell lines used were A6 (derived from toad kidney), LLC-PK₁ (from pig kidney) and MDCK (from dog kidney). These cultures grow with their apical surface uppermost, allowing easy labelling of the apical membrane with fluorescently conjugated lectins. Preparations with exposed basolateral surfaces were obtained by gently detaching part of an intact sheet of cells with a stream of medium from a syringe and folding the flap of cells thus produced back over a glass disk (5 mm diameter, 1 mm high) with the basolateral surface uppermost. This inverted sheet of cells was held in place by a ring clamped around the disk.

Wheat-germ agglutinin and peanut agglutinin lectins were used to label directly both basal and apical surfaces. However, if one surface was labelled, the probe did not redistribute to the other side of the cell, as determined by direct observation with high resolution fluorescence microscopy. It was found that the most convenient geometry for viewing the sidedness of the label was to focus the microscope part way up a dome of cells. Domes are regions of the monolayer which have detached from the dish and bulged upwards due to epithelial transport of solution across the monolayer from apical to basal surface, where it becomes sequestered inside the dome of cells thus produced¹⁶. By focusing part way up the dome, apical and basolateral surfaces on individual cells are in focus simultaneously. Thus, if the entire plasma membrane is labelled, each cell in the cross-section of the dome defined by the plane of focus of the microscope will appear as a complete ring of fluorescence. If only one membrane is labelled, incomplete fluorescent rings, or arcs, will be seen. Figure 1a shows such arcs on a dome of A6 cells having apical membranes stained with rhodamine-conjugated wheat-germ agglutinin.

As the lectin-labelled membrane components were unable to penetrate the tight junction, we next determined whether the

Fig. 2 Molecular structure of the fluorescent lipid probes. AFC₁₆, 5-(N-hexadecanoyl)amino fluorescein; AFC₁₂, 5-(N-dodecanoyl)amino fluorescein (Molecular Probes); diIC₁₆, 3,3'-dihexadecylindocarbocyanine iodide (given by Dr A. Waggoner); NBD-PC, 1-acyl-2-(N-4-nitrobenz-2-oxa-1,3-diazole)-aminocaproyl phosphatidylcholine (Avanti). AFC₁₂, AFC₁₆ and diIC₁₆ did not affect transepithelial resistance when added to the apical solution of A6 epithelia grown on a porous support (Millipore filter).



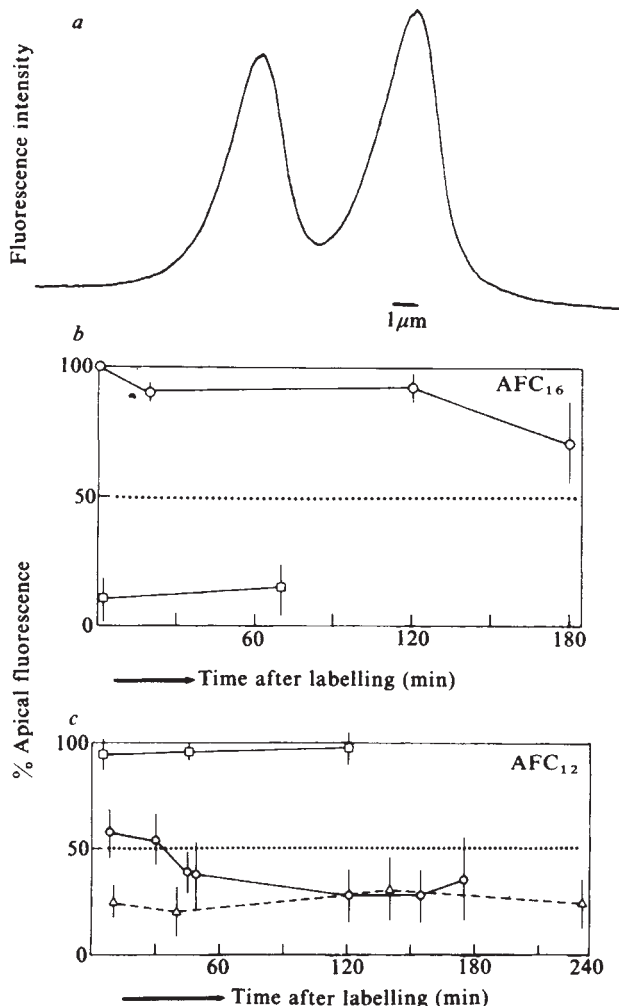


Fig. 3 *a*, Fluorescence intensity (in arbitrary units) excited by focused krypton ion laser beam (at 531 nm) as the microscope focus was scanned through an A6 cell labelled with diIC₁₄. The peak on the left is due to basal membrane fluorescence, and that on the right due to apical fluorescence. *b*, Mean ($\pm$ s.d.) per cent fluorescence excited from apical membrane as a function of time after labelling cells with AFC₁₆. ○, Apical membrane initially labelled; □, basolateral membrane initially labelled. The 476 krypton laser line was used to excite fluorescein fluorescence. *c*, Mean ($\pm$ s.d.) per cent fluorescence excited from apical membrane as a function of time after labelling cells with AFC₁₂. ○, Apical membrane initially labelled; □, basal membrane initially labelled; □, temperature reduced to 10 °C and apical membrane labelled.

tight junction formed a barrier to lateral diffusion of the label in the membrane. Previous measurements of lateral diffusion of wheat-germ agglutinin binding sites on fibroblasts have yielded diffusion coefficients of $\sim 2 \times 10^{-11}$ to 2×10^{-10} cm² s⁻¹ with 80% of the probe freely mobile¹⁷. In contrast, our FPR measurements clearly demonstrated that wheat-germ agglutinin, as well as peanut agglutinin, were completely immobile on both apical and basal membranes. It is thus unnecessary to postulate a diffusion barrier for the maintenance of the asymmetrical distribution of these probes.

The monolayers can be dispersed into single-cell suspensions by chelation of divalent cations, which has been shown to disrupt tight junction filaments in epithelia^{4,6,8,9,11}. When this is done after first labelling one side of the monolayer with a lectin, the label spreads over the entire surface of the cell. After spreading, however, FPR measurements show that the probe is still completely immobile with respect to diffusion. Lectin binding sites maintain their relative positions during the spreading and do not inter-mix. Thus, the movement of the lectin while spreading over the entire cell surface after disruption of the monolayer apparently does not involve diffusion.

Lipid probes

We next investigated the behaviour of several fluorescent lipophilic probes introduced exogenously to the cell membranes. These probes (see Fig. 2) fell into two groups with respect to their labelling patterns: those which penetrated the tight junction (diIC_n for $n = 14, 16, 18, 20$ and AFC₁₂), and those which did not penetrate the tight junction (NBD-PC and AFC₁₆). An example of domes labelled with AFC₁₆ is shown in Fig. 1*b*, where the scalloped pattern due to one-sided labelling is evident. In contrast, Fig. 1*d* shows a dome labelled with diIC₁₆, which labels the entire cell membrane. As was the case with the lectins, all the non-penetrating lipid probes spread over the entire cell surface when the cell monolayer was disrupted by chelation of divalent cations.

We were also able to observe directly diffusion of the diIC_n probes through the tight junction. By carefully manipulating the x - y position of the microscope stage, we manoeuvred a single cell (labelled with one of the diIC_n probes) on the side of a dome (as in Fig. 1*d*) under an intense, focused laser beam so that all the fluorescence from either the apical or basolateral surface was bleached. Then, under normal fluorescence illumination, we observed fluorescence returning to the bleached membrane due to diffusion from the unbleached part of the cell on the other side of the tight junction. The fluorescence was replenished in a few minutes, which is as expected from the measured diffusion coefficient (Table 1). If the entire cell was bleached, no replenishment occurred. There was no transfer of the probe from neighbouring cells.

As the barrier to penetration between apical and basolateral membranes seems to occur at the tight junction, we tested whether epithelial cell lines having different permeability characteristics for aqueous solutes would exhibit different labelling patterns with the lipophilic probes. In many cases, 'leakier' epithelia have less extensive fibrillar networks forming the tight junction than 'tight' epithelia⁵. We found the labelling patterns for the probes in Fig. 2 to be identical in A6 cells (tight epithelium: trans-epithelial resistance = 5,000 Ω cm²), MDCK cells (leaky epithelium: resistance = 100 Ω cm²) and LLC-PK₁ cells (intermediate: resistance = 400 Ω cm²)^{14,18-20}. Thus, for the probes tested, penetrability of the tight junction to probes in the cell membrane does not correlate with tight junction penetrability to aqueous solutes.

A more quantitative measure of the distribution of the fluorescent probes was obtained by measuring fluorescence intensities from apical and basal membranes separately on individual cells. The focused laser beam was used to excite fluorescence from a small spot (as in the FPR measurements) as the microscope focus was slowly scanned through the centre of a cell. A peak of fluorescence appeared, first as the apical, then as the basal membrane passed through focus (Fig. 3*a*). Figure 3*b* shows the time dependence of the fluorescence distribution thus measured for AFC₁₆. This probe clearly does not penetrate significantly from apical to basal membrane, or vice versa, over time periods of at least 2 h. AFC₁₂, on the other hand, distributes

Table 1 Lateral diffusion coefficients of lipid probes in A6 cell membranes

Probe	Apical	Basal	Pass tight junctions
diIC ₁₄	9.7 $\pm$ 3.6 (17)	11.9 $\pm$ 2.3 (14)	+
diIC ₁₆	7.9 $\pm$ 3.4 (18)	10.2 $\pm$ 5.6 (19)	+
AFC ₁₂	13.3 $\pm$ 4.6 (10)	11.2 $\pm$ 3.2 (9)	+
AFC ₁₆	4.2 $\pm$ 1.8 (18)	ND	-
NBD-PC	2.9 $\pm$ 1.9 (33)	4.2 $\pm$ 1.7 (28)	-

Values shown are the mean lateral diffusion coefficient (D) $\pm$ s.d. ($\times 10^9$ cm² s⁻¹). The number of cells measured is shown in parentheses. ND, not determined. The values for D are not corrected for the non-planar membrane geometry due to the presence of microvilli, which probably accounts at least in part for the higher values usually obtained on the basal membranes, which are non-villous.

so that ~30% of the fluorescence finally localizes on the apical membrane, regardless of whether the apical or basal membrane was labelled initially (Fig. 3c). In addition, the distribution of AFC₁₂ is temperature dependent, with no passage from apical to basal membranes occurring if the cells are kept below 10°C—this cannot be explained by a low temperature immobilization of AFC₁₂, as FPR measurements at this temperature showed that AFC₁₂ was still free to diffuse, although at a rather slower rate (data not shown).

The time course of the AFC₁₂ distribution has another curious feature. Within minutes after labelling the apical membrane, the probe redistributed so that 50–60% of the fluorescence localized on the apical membrane. Within the next 30–60 min this proportion gradually changed until only 25–30% of the fluorescence lay on the apical surface (Fig. 3c). This is not a trivial geometric effect due to the presence of more membrane on the basal surface. In fact the opposite is the case¹⁴. The same apportioning of fluorescence occurred when the intact cell monolayer was detached and turned over and the basolateral membranes labelled initially (Fig. 3c). In contrast, the diIC₁₆ maintains 50–60% of its fluorescence on the apical membrane.

It is possible that the lipid compositions of the apical and basolateral membranes are different, and that AFC₁₂ prefers to 'dissolve' or partition into the lipids of the basolateral membrane. This is plausible, as differences in lipid composition between apical and basolateral membranes of epithelia have been reported^{3,13,21–23}. Another possibility is that the probe's fluorescence is partially quenched at the apical surface, perhaps, for example, by low pH. We have no evidence to exclude either possibility.

Basis of selectivity of the tight junction barrier

The molecular basis for the selectivity of the tight junction barrier to penetration of lipid probes is of primary importance both because of what it may reveal about the structure of the tight junction itself, and because of the implication that it may permit the cell to maintain different lipid compositions on the apical as opposed to the basolateral membranes. From the structures shown in Fig. 2, it is evident that molecular charge cannot be the sole determining factor, as there are positively and negatively charged probes which do penetrate (diIC_n and AFC₁₂), and negatively charged and uncharged probes which do not penetrate (AFC₁₆ and NBD-PC) the tight junction. The degree of partition of the probe between aqueous and membrane phases is also probably not important in determining whether the probe penetrates the tight junction. AFC₁₂ and NBD-PC are the two probes with the highest aqueous phase partitioning, but they exhibit opposite behaviour with respect to passing through the tight junction, as do AFC₁₆ and the diIC_n, which partition least in the aqueous phase.

It is interesting that AFC₁₂ and AFC₁₆, which differ only in fatty acyl chain length, show opposite behaviour regarding penetration of the tight junction, which may indicate that interactions in the hydrophobic region of the membrane are important. In this regard, Table 1 shows that the lateral diffusion coefficients of the lipid probes are predictive of whether or not the probe penetrates the tight junction: all probes that can pass through the tight junction have lateral diffusion coefficients greater than $\sim 8 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$, whereas all those that are incapable of penetrating the junction have lateral diffusion coefficients $< 4 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$. Apparently, the two classes of lipid probes interact with the lipid bilayer in significantly different ways, as reflected by their lateral diffusion coefficients. This difference may provide the basis for whether a probe can penetrate the tight junction or not.

In view of the possibility that the two classes of lipid probes interact differently with the cell membrane, it is important to assess whether the probes are in fact inserted into the membrane lipid bilayer. From the molecular structures in Fig. 2 one would expect, on energetic grounds, insertion of the hydrocarbon chains into the membrane bilayer. On the other hand, it is

conceivable that a probe may form micellar structures which adhere to the cell surface and prevent true incorporation of the probe into the membrane. We consider this unlikely for several reasons. First, the measured diffusion coefficients are all in the range observed for lipid-like molecules in cell membranes^{24–29}. In addition, the conjugated bridge of diIC₁₈ has been shown to be aligned parallel to the plane of the membrane when the probe labels erythrocyte ghosts, exactly as expected if the hydrocarbon tails are inserted into the membrane bilayer³⁰. Finally, our fluorescence intensity and spectral measurements have shown that the probes are in a much more hydrophobic environment when labelling cells than when dispersed in aqueous media³¹, so that the possibility of probe micelles adhering to the cell surface is unlikely.

We feel that a possible basis for the observed selectivity of the tight junction lies in which leaflet of the membrane bilayer is occupied by a given probe; those probes which label the outer leaflet only do not pass through the tight junction, whereas those which can 'flip-flop' to the inner leaflet do pass through it. Phospholipids have been shown to flip-flop between membrane leaflets at very low rates in bilayer membranes^{32–34}, and it seems reasonable to assume that NBD-PC behaves similarly. The smaller polar 'head group' region of the diIC_n may allow it to pass more rapidly between inner and outer membrane leaflets, and the shorter acyl chain of AFC₁₂ relative to AFC₁₆ may facilitate its flip-flop, a process which is inhibited at low temperature.

Experimental evidence in support of this hypothesis has come from measuring the accessibility of the various membrane-bound probes to quenching from outside the membrane³¹. Using iodide to quench diIC₁₆ fluorescence and anti-fluorescein antibodies to quench AFC₁₂ and AFC₁₆ fluorescence, our data show that (1) diIC₁₆ seems to have access to both leaflets of phospholipid vesicle membranes; (2) AFC₁₆ is present almost exclusively in the outer membrane leaflet of labelled cells; and (3) AFC₁₂ can 'flip-flop' between membrane leaflets at 20 °C, but not at 5 °C. These results imply that the tight junction may form a barrier to passage of lipids only in the outer membrane leaflet. Thus, differences in lipid composition between apical and basolateral membranes could reflect differences in the compositions of the outer monolayer lipids.

Conclusion

We have studied the mechanism of maintenance of cellular asymmetry in epithelial cells grown in culture by directly observing whether fluorescent probes applied to either the apical or basolateral membranes spread to the unlabelled surface. Lectins which were bound to either the apical or basolateral membrane remained segregated there and were immobile with respect to lateral diffusion in the membrane. Some lipid probes introduced exogenously into the membrane were able to penetrate to the unlabelled side of the cell monolayer, whereas other lipid probes were not. All lipid probes were freely mobile in the membrane, although those which were able to spread over the entire cell had lateral diffusion coefficients at least twofold greater than those which could not. The barrier to spreading of the probes to the unlabelled surface was at the region of the tight junction. Chelation of divalent cations, which disperses the monolayer and disrupts tight junction filaments, allowed spreading of all the impermeant probes over the cell surface, although in the case of the lectins the spreading apparently did not involve free diffusion. The selectivity of the tight junction region to passage of the lipid probes was identical in monolayers of epithelial cell lines having markedly different 'leakiness' to aqueous solutes.

These results directly demonstrate that the region of the tight junction can act as a barrier to lateral diffusion of membrane constituents as small as lipids, potentially enabling epithelia to segregate at least some lipids to the apical or basolateral membrane and thus maintain an asymmetric lipid distribution. Fluorescence quenching experiments support the hypothesis that the lipid probes that have access to the inner leaflet of the

cell bilayer are capable of passing the tight junction, but those present exclusively in the outer leaflet are blocked by the tight junction.

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LETTERS TO NATURE

Probable optical counterpart of a γ -ray burster

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I describe here 1.6×10^7 s (over half a year) of optical monitoring of three γ -ray burst positions using the collection of archival plates at the Harvard College Observatory. The search has uncovered the probable optical counterpart for the 19 November 1978 γ -ray burster on a blue emulsion plate exposed in 1928. The optical flash observed on the 1928 plate has $m_B \sim 3$, for an assumed duration of 1 s. Optical searches indicate that the absolute magnitude of the γ -ray burst system at quiescence is fainter than 13. A recurrence rate of $\sim 10^{-7.5} \text{ s}^{-1}$ is found from the γ -ray and optical data. A recurrence rate this high rules out any model which uses a collision between a neutron star and an asteroid-like body as well as any model which requires accretion from the interstellar matter onto a neutron star.

Since the discovery of cosmic γ -ray bursts¹, there have been many observations of these phenomena², but so far no celestial object has been positively identified with any γ -ray burster at other than γ -ray energies^{3–7}. In order to make an identification, an interplanetary network of satellites with γ -ray detectors has been established, so as to locate the direction of the γ -ray burst by triangulation^{2,3}. Although this network has determined error boxes ~ 1 arc min in radius for several bursters^{3,8–11}, no convincing optical^{3,12,13}, radio¹⁴ or X-ray^{10,15} counterparts have been proposed.

Grindlay, Wright and McCrosky⁷ have shown that a simple extrapolation of the observed γ -ray spectrum implies that the sources would be optical flares with $m_v \sim 0$. This failure to detect optical flashes from two γ -ray bursts in the early 1970s allowed them to set limits on the brightness of the two events as $m_v > 3.0$ and 3.8 mag for an assumed duration of 1 s. The advantage of their work over that reported here is that a γ -ray burst was seen during their exposure.

Both theoretical and observational arguments suggest that γ -ray bursters are recurring phenomena. Jennings and White¹⁶ use the observed $\log N(>S) - \log S$ relation to deduce that the γ -ray recurrence rate must be $>10^{-12} \text{ s}^{-1}$ per burster. For thermonuclear flash models¹⁷, the recurrence rate is predicted to

be between 10^{-10} and 10^{-7} s^{-1} . The observational argument is based on several bursts, closely spaced in time, which seem to have identical positions^{18–20}.

I have carried out a search of three precisely known γ -ray burst positions^{8–10} on plates in the archival collection of Harvard College Observatory (see Table 1). On one of the plates for the 19 November 1978 field¹⁰, a new 10-mag star appears in the γ -ray error box. The plate is MF12559 taken on 17 November 1928 in South Africa. It is the fourth of six identical 45-min exposures taken in succession. Figure 1 is a reproduction of the third and fourth plates in the series. The 'new' star does not appear on any of the other five plates (all of which have a limiting magnitude of ~ 15).

Microdensitometry provides strong evidence that the 'new' star image was formed by a brief flash of light which came through the telescope. The images on the fourth plate are trailed by 17 arc s, but the image of the 'new' star is not noticeably trailed, as would be expected if it were a short (≤ 10 min) optical flash. Figure 2 shows the profiles of the burst image and of two nearby normal star images in a direction perpendicular to the trailing. The asymmetry is due to coma, which implies that the burst image must have been formed by light that passed through the telescope optics and that plate defects and light leaks are not a possible explanation for the burster candidate. The profiles of normal stars as well as the candidate show a break at $\sim 110 \mu\text{m}$, which is further confirmation that the light that formed the burst image followed the same path as normal starlight.

The slope of the burst image profile in Fig. 2 is less than the slope of a normal star profile, which can be attributed to the failure of the law of reciprocity in photographic emulsions. In the relevant range of radial distances (r), the intensity (I) of light that reaches the plate will fall off as $r^{-\eta}$ where η is some constant^{21–23}. According to Schwarzschild's law of reciprocity failure^{24–26}, the photographic density (D) is proportional to $I^q T$, where q is a function of the duration of exposure (T). Thus $D \sim r^{-\eta q}$ and the slope on a $\log D$ versus $\log r$ plot is $(-\eta q)$. For a short flash, q is ≤ 1 (ref. 26). For a 45-min exposure, q is typically²⁶ 1.25, so that the normal star profile is expected to be steeper than the profile from a quick flash. Unfortunately, this argument cannot be made more quantitative because the emulsion type and developing conditions are unknown.

The 1928 image may have been formed by a 2-mag meteor viewed exactly head-on. The probability²⁷ of detecting a meteor in a γ -ray error box on any plate used in this study is $\sim 10^{-2}$. Microdensitometry shows that the 1928 image is not trailed,

with an upper limit of 3 arc s trailing. The probability that a meteor will approach within this narrow tolerance of the head-on direction is 10^{-8} . The joint probability of both seeing a meteor in a γ -ray burst error box and having that meteor appear head-on is $\sim 10^{-10}$.

The position of the image (1 h 16 min 25.8 s $\pm$ 0.6 s and $-28^\circ 51' 1.2'' \pm 2.1''$ measured with a Mann measuring machine) is inside the γ -ray error-box¹⁰ but is not consistent with any observed radio sources¹⁴ (see Fig. 1). The position is, however, inside the reported error-box of a possible (3.5σ) X-ray source¹⁰. Recently, M. H. Liller took deep *B* and *V* 4-m plates of this region at CTIO. Both plates show faint stellar images ($m \sim 23$) at the same location in the optical error box

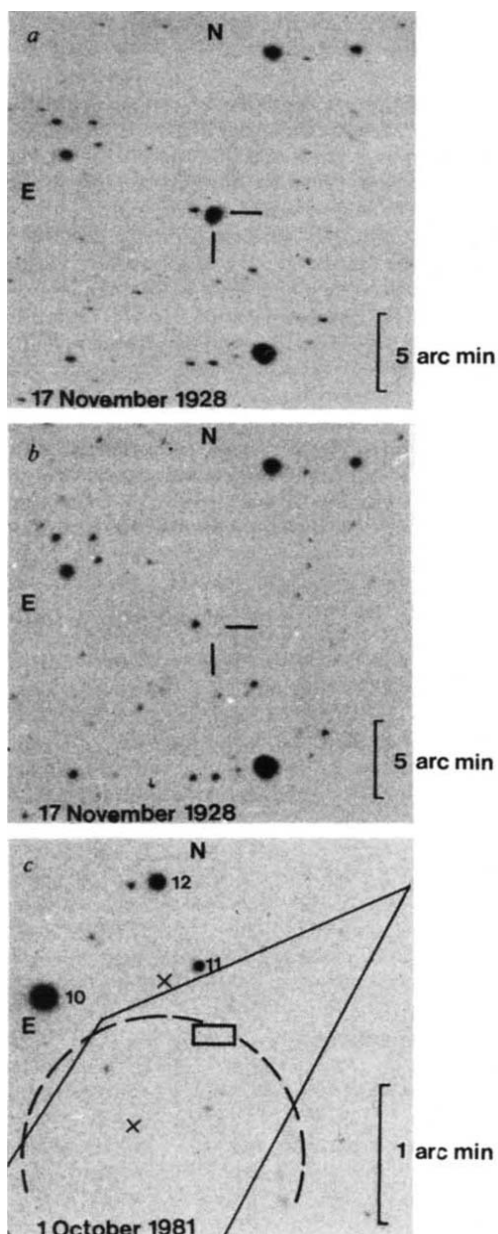


Fig. 1 Three views of the 19 November 1978 γ -ray burst field. *a*, Plate MF12559, with tick marks indicating the burst image. The burst image has a different shape from that of a normal star; a short flash is not trailed and has a more gradual fall off of density with distance from the centre of the image. *b*, Plate MF12558, which was taken 45 min before MF12559. *c*, A *V* plate taken by M. H. Liller on the 4-m telescope at CTIO. The image inside the 2σ 1928 optical positional error rectangle (centre) cannot be seen. The γ -ray error region¹⁰ (solid lines) and possible X-ray error circle¹⁰ (dashed lines) are also shown. Radio sources¹⁴ are indicated with crosses. Three stars are numbered as in ref. 12.

Table 1 Exposure versus burst limiting magnitude

Limiting mag. for detecting a 1 s burst	Hours of exposure which reach the limiting mag			
	19 November 1978	5 March 1979	6 April 1979	Total
3.0–3.9	671	2,270	1,509	4,450
4.0–4.9	635	2,148	1,422	4,205
5.0–5.9	560	1,716	1,198	3,474
6.0–6.9	407	961	710	2,078
7.0–7.9	289	373	269	931
8.0–8.9	174	149	71	394
9.0–9.9	96	26	23	145
10.0–10.9	34	1	7	42

(1 h 16 min 25.99 s $\pm$ 0.02 s and $-28^\circ 51' 2.3'' \pm 0.3''$, 1950.0). Using a PDS microdensitometer, I find the images to be 3.2σ and 4.9σ above noise for the *B* and *V* plates respectively. A search by G. R. Ricker (personal communication) has, however, failed to locate this star with a CCD camera (the MASCOT) on the McGraw-Hill 1.3-m telescope ($m \geq 24$ at 2σ for 4,000–7,000 Å). Because of the uncertain thresholds and different band-passes, the question of the reality of this stellar image is not yet established. Deeper searches with the MASCOT have been carried out and the data are being analysed.

The magnitude of the image on the 1928 Harvard plate is uncertain because the profile of the burst image differs from a comparison star's profile. Exact modelling is impossible because the plate characteristics and burst duration are unknown. However, a reasonable model shows $m_B \sim 3$ for a burst lasting 1 s, which corresponds to a time-integrated flux of 4×10^{-7} erg cm⁻² above the atmosphere²⁷. Assuming that the 1928 and 1978 bursts were similar, $L_\gamma(>30 \text{ keV})/L_{\text{opt}}(B \text{ band})$ is 800 and the energy flux density is proportional to $\nu^{0.6}$.

Three other plates show density enhancements in the γ -ray burst error regions. A plate exposed in 1948 shows a 'new' star in the 19 November 1978 optical error box, and two plates (from 1932 and 1936) show 'new' stars in the 6 April 1979 γ -ray error box. Because these three images are near the limiting magnitude of their respective plates a detailed analysis of the image profile is impossible.

Table 2 summarizes the characteristics and total exposure for the seven series of plates used in this study. For each plate, I estimated the magnitude of the faintest star image detectable. 'Burst-limiting magnitude' for each plate was derived from the known exposure times, an assumed burst duration, an approximate knowledge of the reciprocity failure, and the stellar limiting magnitude. The burst-limiting magnitude indicates how bright an optical flash of given duration must be to ensure detection on a given plate. An optical flash inside the nebosity of N49 would be harder to detect, so the 'burst limiting magnitude' should be decreased by up to 1 or 2 mag for the 5 March 1979 field. I assume that the optical burst lasts 1 s as this is comparable to the durations of γ -ray bursts. Table 1 shows the total exposure for each of the three γ -ray positions that could lead to the detection of a burst of a given magnitude.

The steady optical flux from the burster can severely limit the types of stellar components which can be in the burster system. The CTIO 4-m plates of Liller indicate that the quiescent optical counterpart is fainter than ~ 23 mag. Jennings and White¹⁶ show that the γ -ray bursters are probably not extragalactic nor are they distributed in a halo around our Galaxy. They deduce that the most likely distribution of γ -ray bursters is a disk population with a scale height of ~ 400 pc. In this case, the distance to the 19 November 1978 burster must be ≤ 1 kpc, because of its position near the south galactic pole. The limits on both the apparent magnitude and the distance yield a lower limit of 13 mag for the burster's absolute magnitude. Only three known classes of stars (white dwarfs, neutron stars and late M main sequence stars) satisfy this limit, and hence could be components of the burster system. Furthermore, if the ~ 23 mag star on Liller's plate is the optical counterpart of the burster, then this cannot be a lone neutron star. This is because a lone neutron star has an absolute

Table 2 Characteristics of Harvard plates

Plate series	Aperture (cm)	Plate scale (arc s mm ⁻¹)	Dates	Total exposure (h)	Total no. of plates	Median limiting mag.	Median limiting mag. for 1 s burst
A	61.0	60	1896–1950	68	50	16.5	8.2
AM/AX	3.8	600	1901–1953	2,689	2,465	14.1	5.8
B	20.3	179	1889–1948	198	414	15.3	8.1
Damon	4.1	580	1970–1979	75	50	15.2	6.9
MF	25.4	167	1918–1950	210	299	16.4	8.9
RB	7.6	391	1928–1950	1,210	857	14.8	6.5

magnitude >23 (assuming the neutron star radiates as a black body²⁸), and would have to be placed at such a close distance as to violate the $\log N(>S) - \log S$ observations. We note (D. Q. Lamb, personal communication) that the class of γ -ray burst models which invoke an energy supply internal to a neutron star²⁹ do not require that the neutron star have a companion.

The value for the burster recurrence rate can be estimated. (1) From detailed modelling of the $\log N(>S) - \log S$ relation, Jennings and White¹⁶ show that the recurrence rate must be between 10^{-12} and 10^{-1} s. (2) Let N_T be the total number of γ -ray burst sources which are accessible to near-Earth detectors with a threshold f_0 , and let R be the number of γ -ray bursts observed with flux $>f_0$ which arrive at Earth per second. The average recurrence rate will be just R/N_T . N_T must be greater than the total number (N_0) of non-overlapping error boxes determined for bursts with a flux greater than f_0 . From the data base of Mazets and Golenetskii²⁰, with $f_0 \sim 10^{-6}$ erg cm⁻², $R \sim 10^{-5.8}$ s⁻¹ and $N_T > N_0 > 35$, the recurrence rate must be $<10^{-7.3}$ s⁻¹. (3) With a total observing time of $10^{7.3}$ s, the data from Mazets and Golenetskii²⁰ suggest two and possibly more cases of a γ -ray burster recurring. These cases suggest the burst

rate is $\sim 10^{-7}$ s⁻¹. (4) One (and possibly as many as four) optical bursts were seen during a monitoring time of $10^{7.2}$ s, which implies a recurrence rate of order $10^{-7.2}$ s⁻¹. From all the evidence, a reasonable estimate of the burster recurrence rate is $\sim 10^{-7.5}$ s⁻¹ (one burst per year).

This high recurrence rate precludes the hypothesis where an asteroid (or comet) impacts onto a neutron star³⁰, which predicts typical recurrence rates on the order of 10^{-13} s⁻¹ (refs 31, 32). A popular class of models envisions a thermonuclear explosion on a neutron star surface. For a typical burst energy¹⁶ of 10^{39} erg and mass-to-energy conversion³³ of 10^{19} erg g⁻¹, the neutron star will need to accrete 10^{20} g of fuel for each burst. To transfer this amount of mass every $\sim 10^{7.5}$ s, the average accretion rate is many orders of magnitude higher than is obtainable by accretion from the interstellar medium alone. If this accretion rate remains constant, then a source 100 pc distant would imply a steady X-ray flux of $\sim 10^{-10}$ erg s⁻¹ cm⁻². Observations with the Einstein Observatory³ have placed upper limits of $\sim 10^{-12}$ erg s⁻¹ cm⁻² on the steady X-ray flux from the three γ -ray bursters listed in Table 1. If the thermonuclear explosion model and the physical values are correct, this discrepancy can be explained by assuming either that most of the accretion energy is not radiated in the X-ray or that the accretion is not steady.

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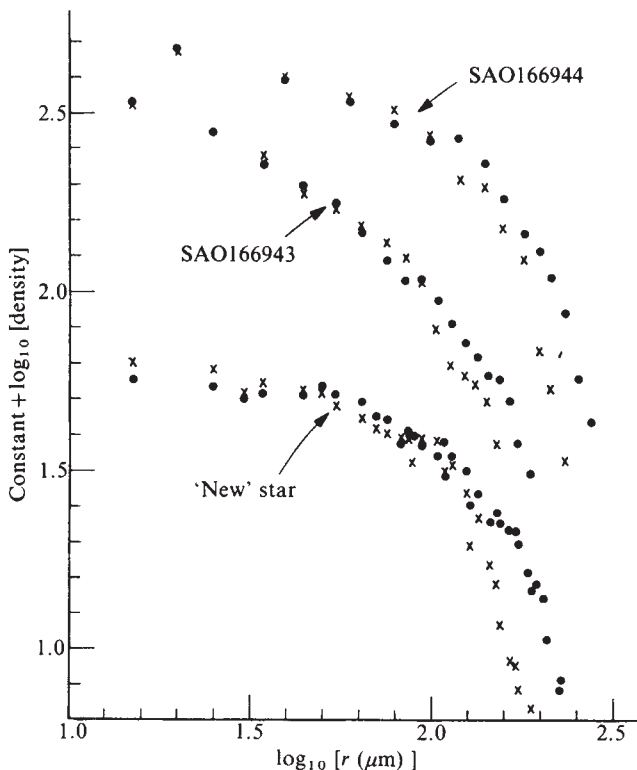


Fig. 2 Profiles along the north-south axis of the burst image and two nearby normal stars. The densities north (south) of the image centre are indicated with $\times$ ($\bullet$). The differences between the north and south profiles are due entirely to coma, because the scan direction is perpendicular to the direction of trailing. The profile of the burst image has been lowered by 0.6 in the logarithm of density for clarity. The zero of the density scale is set to the density of the sky background.

A four-hour orbital period of the X-ray burster 4U/MXB1636-53

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It has been generally accepted that X-ray burst sources and other non-bursting 'galactic bulge X-ray sources' are low-mass close binary systems (for a review see ref. 1). The companion stars of the transient burst sources Aql X-1 (= Aql MXB = 4U1908+00) and Cen X-4 (= XB1455-31) are of spectral types G7-K3V (ref. 2) and K3-7V (ref. 3), respectively. The companion of the non-bursting transient source A0620-00 is of spectral type K4-5V (refs 4, 5). If these stars fill their Roche lobe, which is likely, the orbital periods are in the range 5-8 h. There is some evidence from X-ray observations alone that the orbital period of Cen X-4 is ~8 h (ref. 6). From an analysis of their average optical properties, one of us (J.v.P. ref. 7) estimated that the typical values of the companion star masses and orbital periods of this class of low-mass X-ray binaries are ~0.6 $M_{\odot}$ and ~6 h, respectively. The orbital period recently suggested for the burst source 4U/MXB1735-44 (ref. 8) is in accordance with this general picture. We here report variations in the persistent optical flux of 4U/MXB1636-53 which seem to vary periodically with a period near 4 h.

During June-August 1979 and 1980 extensive optical observations were made of the X-ray burst source 4U/MXB1636-53 (see refs 9, 10 for properties of the faint blue counterpart ($B \sim 18$) of this source). Some of these observations were made simultaneously with the Japanese X-ray observatory Hakucho. Results on correlated optical/X-ray bursts and other optical bursts will be reported elsewhere^{11,12}.

The observations were made with the Danish 1.5-m telescope at the European Southern Observatory (ESO) at La Silla. During the 1980 observations a double-channel photometer was used with no filter. The combination of atmospheric transmission, optics and photomultiplier sensitivity defines a 'white light' passband with an effective wavelength of ~4,300 Å and a full width at half-maximum of ~1,900 Å. The telescope was guided using an autoguiding system which kept the star centred to ≤ 1 arc s. In the channel used for measurements of 4U/MXB1636-53, a 5 arc s diaphragm was used. The reference channel which used a 20 arc s diaphragm was set on a star ~3 mag brighter a few arc minutes away from the main channel. The ratio between stellar signal and sky brightness was similar for the two channels (1.5 and ~1.2, respectively).

We observed 4U/MXB1636-53 on 11-13 July 1980. The data trains span ≈ 7 h each night. The nights were dark, photometric and the seeing was less than 2 arc s. Figure 1 shows the observed count rates binned into 512-s time slots, together with similar data for the reference channel. The latter is constant to within $\pm 2\%$ throughout each night. Clearly, however, the persistent optical flux of 4U/MXB1636-53 is varying. As the ratio of stellar to sky signal is approximately equal in the two channels, this variation cannot be due to changes in the sky brightness. Such changes would result in a similar variation (approximately same percentage) in the reference signal and that is not observed. Furthermore, the relatively good seeing in combination with the 5 arc s diaphragm and the high-quality autoguiding of the star (see above), exclude the possibility that

the variation is due to variable signal contamination by a star of similar brightness about 7 arc s north of 4U/MXB1636-53. The data suggest that the variability may be periodic. Five broad maxima occur in the optical brightness near 11 July, 0200 and 0615 UT, 12 July, 0100 and 0430 UT and near 13 July, 0315 UT. These maxima are consistent with a single period of approximately 4 h.

To establish a more accurate period, we have folded the data with trial periods between 2 and 6 h, and searched for periods where χ^2 of the data points, relative to the average curve (divided into 20 phase bins), showed a minimum. Before folding the data, the overall nightly average counting rate was subtracted. Three possible periods turned up (see Fig. 2) 3.28 ± 0.05 h, 3.78 ± 0.05 h and 4.42 ± 0.05 h, corresponding to 15, 13, and 11 cycles between the maxima on 11 July, 0200 UT and 13 July, 0315 UT, respectively. Figure 3 shows the folded count rates (as described above) for the period of 3.78 h. The amplitude is ~25% of the average signal (sky background subtracted).

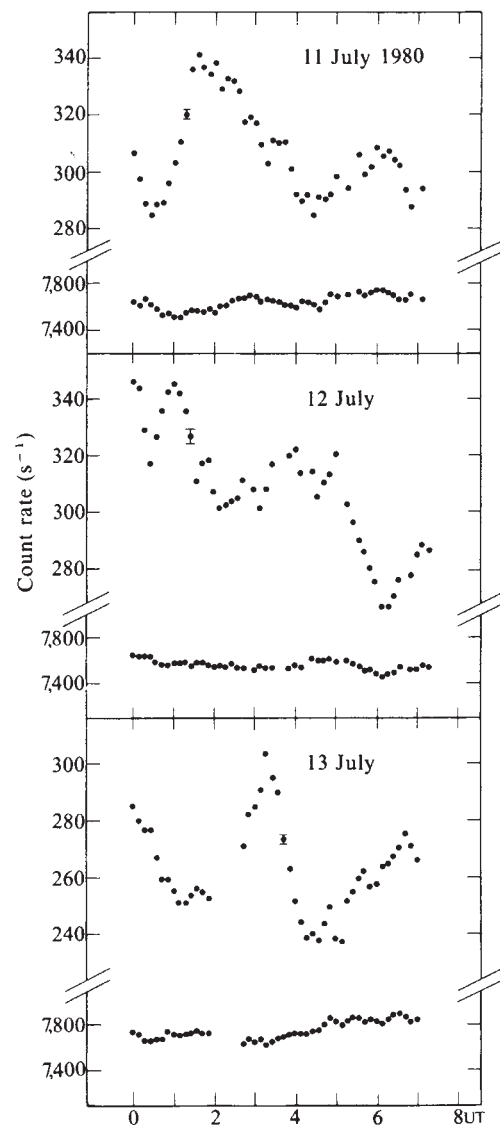


Fig. 1 Count rates, including sky background, for 4U/MXB1636-53 and the reference star (upper and lower set of points in each panel) as observed on 11, 12 and 13 July 1980. Each point represents the average of 10 integrations of 51.2 s each. Typical mean errors on the averages of these 10 values are indicated in the figure by one error bar in each panel. The error bars in the reference star data have approximately the size of the dots. The blue magnitude corresponding to the average sky-corrected counting rate is $B \sim 18.5$.

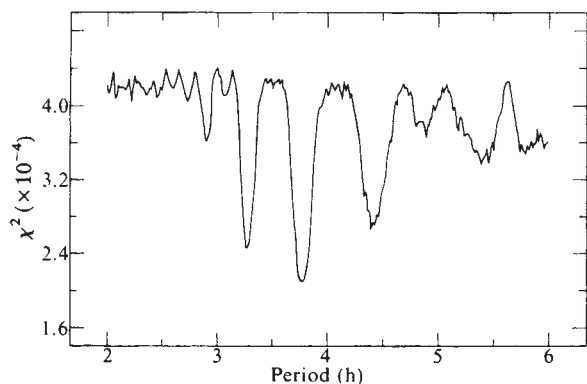


Fig. 2 χ^2 (relative to the average folded curve divided into 20 phase bins) as a function of the folding period. The three deep minima correspond to periods of 3.28, 3.78 and 4.42 h, respectively.

We observed 4U/MXB1636–53 again on 1 August 1981, 0140 UT until 0445 UT, again using the Danish 1.5-m telescope. The photometer used was operated as a single channel instrument. The detector was an RCA C31034A photomultiplier tube illuminated through a 5-mm liquid CuSO_4 filter. A 9 arc s diaphragm was used. The observations were repeatedly interrupted to measure the sky background and the brightness of two nearby companion stars. Photometric conditions were good, with seeing of less than 2 arc s. To avoid any possible contamination of the signal by light from the star ~ 7 arc s north of 4U/MXB1636–53, the centre of the diaphragm was placed about 1.5 arc s south of 4U/MXB1636–53.

Figure 4 shows the ratio of the counting rates (sky background subtracted) of 4U/MXB1636–53 to one of the reference stars. A smooth modulation of the brightness of 4U/MXB1636–53 is clearly visible, with a shape that is consistent with the average variation found in the 1980 observations (see Fig. 3). Clearly the data are insufficient to decide between the above three allowed time periods.

If any of these three possible periods is the orbital period of 4U/MXB1636–53, then the mass of its companion star can be estimated if the following assumptions are made (see refs 13, 14). (1) The companion star fills its Roche lobe. This is likely in view of the rate of mass transfer needed to power the persistent X-ray emission of 4U/MXB1636–53. (2) The companion star is on the main sequence.

The orbital period, P , is then uniquely determined by the mass M_c of the companion star according to the relation $P = 3.14 \times 10^4 \times (M_c/M_\odot)$ s. Using this relation and the above

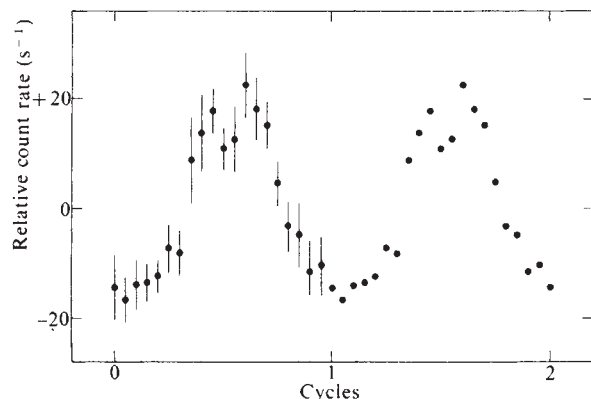


Fig. 3 Average light curve of 4U/MXB1636–53 during 1980, 11, 12 and 13 July, for a folding period of 3.78 h. Before folding the data, the average counting rate, as observed during each night, has been subtracted. The error bars indicate the mean errors of the average values in the phase bins. For convenience the same data have been plotted twice.

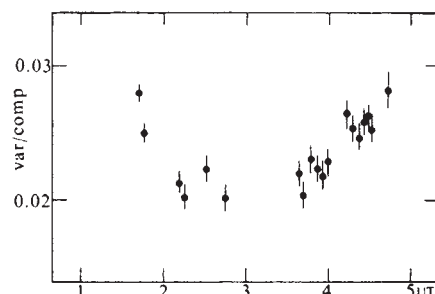


Fig. 4 Ratio of the counting rates (sky-subtracted) of 4U/MXB1636–53 (var) to that of one of the comparison stars (comp). The corresponding ratio of the two comparison stars used is constant to within $\pm 2\%$. The data were taken on 1 August 1981.

period of ~ 4 h, we find a mass of $\sim 0.4 M_\odot$ (corresponding to an M0 V star) for the companion of 4U/MXB1636–53.

If we identify the period in the optical brightness variations of 4U/MXB1636–53 with the binary period, a major deviation from axial symmetry must be present in the system. Within the framework of the low-mass X-ray binary model we can exclude the following possible causes of the brightness modulation.

(1) Eclipses of the accretion disk by the companion star (assumed to fill its Roche lobe). They would result in a much narrower minimum in the light curve than is actually observed (see Fig. 3). (2) As the companion star contributes only of the order of 1% or less to the total optical brightness of the system⁷, changes in its visibility cannot explain the observed brightness modulation either. In particular double-wave ellipsoidal variations (with a corresponding period near 8 h) can be excluded. (3) The modulation cannot be the result of eclipses of a hot spot (where matter is injected into the disk), as observed for some cataclysmic variables¹⁴. The luminosity of such a hot spot is expected to be approximately independent of the nature of the compact star at the centre of the disk (white dwarf or neutron star). Because the optical luminosity of low-mass X-ray binaries is a factor of $\sim 10^2$ larger than that of cataclysmic variables this spot would therefore only contribute of the order of 1% of the total optical brightness of the system.

The disk thickness (seen from the compact object), estimated on the basis of the average optical and UV properties, is $\geq 6^\circ$ (ref. 7). In a binary system with a mass ratio $q = 0.3$ and total disk thickness $\geq 6^\circ$, the solid angle subtended by the non-shielded part of the companion star (as seen from the compact object) is $\sim 30\%$ of that of the disk. Thus, if the accretion disk in 4U/MXB1636–53 has a total thickness of about 6° and is not thicker, a significant contribution to the optical brightness variation might be due to the heating by X rays of the companion star and the observed modulation could then be due to the orbital motion of the companion star. It may be that the accretion disk in this system is much thicker than 6° (see the suggestion in ref. 15). For total disk thickness of $\sim 30^\circ$ the optical companion would lie entirely in the X-ray shadow of the disk and this could naturally explain why X-ray eclipses are, in general, not observed in these systems^{1,15}. If such a thick disk is present in 4U/MXB1636–53, we suggest that this disk is not axially symmetric, perhaps being thicker where matter is injected into the disk. A similar suggestion has been made for the source 2A1822–37 (refs 16, 17). The brightness modulation could then be the result of the azimuthal dependence of the surface brightness of the disk in combination with the phase dependence of the angle at which the different parts of the disk are viewed.

If this is the case and also if the observed modulation is due to X-ray heating of the companion, we expect a similar modulation with the orbital period of the average ratio of optical to X-ray burst energies. Clearly we then expect that the observed delay between optical and X-ray bursts will also vary with the ~ 4 h period.

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Intrinsic variations of the double quasar 0957+56 AB

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Spectroscopic evidence strongly suggests that the two quasars 0957+56 A and B are images produced by the gravitational lensing of a single distant quasar (at $z = 1.41$) by a giant elliptical galaxy and its attendant cluster (at $z = 0.36$)^{1–6}. It has also been suggested that the quasar is an intrinsic variable^{7,8}. I report observations that show a variation of ~ 1 mag in both components and behaviour typical of intrinsically variable quasars with similar radio structure. The near constancy of the magnitude difference between the components at several epochs despite overall variations favours all the variations being intrinsic to the quasar and also supports the gravitational lens hypothesis. Recent variations of component B1 limit the time delay between the images to between 2 and 24 yr.

In an analysis of the imaging of the double quasar Young *et al.*⁵ suggested that three images are formed. Two images A and B1 lie 5 and 1 arc s respectively on either side of a faint elliptical galaxy and correspond to the objects for which photometry and spectrophotometry have been reported. The weak third image B2 is so far undetected and thought to be coincident with the galaxy. Photometry of components A and B1 were obtained from the Palomar Observatory Sky Survey (POSS) and the 26-inch refractor at Herstmonceux. The new photographic B band observations are combined with magnitude differences from photometry and spectrophotometry given by various authors: despite the variety of systems used the observations should be directly comparable. Details of the reductions are given in Fig. 1 legend.

Figure 1 shows the light curve of the double quasar between April 1979 and June 1981. The Herstmonceux photometry is consistent with component A being constant and Young *et al.*⁵ also found A constant before the Herstmonceux observations at $B = 17.6$. The observations of component B1 are in excellent agreement and show that from April to December 1979 both components were constant with B1 ~ 0.35 mag fainter than A. Between December 1979 and June 1980 B1 brightened by ~ 0.45 mag and between June 1980 and June 1981 it faded slightly. If this downward trend continues component B1 will return to its previous luminosity by $\sim$ April 1982. Notice that the rise and decline do not have the same slope.

Although all the observations of component B1 are to some extent contaminated by the elliptical galaxy and the weak third image B2 the effects are probably negligible. The contribution from the galaxy decreases rapidly towards the blue and in the B

band is $\leq 5\%$ (ref. 4). This small additional flux will be essentially constant for all observations employing an aperture of a few arc seconds. The magnitude differences from the spectrophotometry refer to the continua while the B band photometry includes the C III] λ 1,909 line. However the line contributes $\leq 5\%$ and will be essentially the same for both components and the line strength is not expected to vary⁹. The third image B2 contributes $\leq 5\%$ to B1 so its effect will also be negligible⁵.

As for longer term changes the double quasar appears on adjacent POSS plates taken in February 1953 and January 1955. From the O-plates the B mag of component A is 16.9 and 16.6 ± 0.2 on the two dates respectively, with component B1 ≈ 0.3 mag fainter on both occasions. So both components were ~ 1 mag brighter and the magnitude difference about same as in 1979. From archival plates taken in 1902, 1903 and 1915 Keel⁸ finds both components ~ 0.5 mag brighter than at present and the magnitude difference is ≈ 0.5 .

The near constancy of the difference despite an overall 1 mag variation strongly suggests that one component follows the other as would be expected with intrinsic variations and that the time delay is short compared with the intervals between epochs. 0956+57AB can be compared with quasars of similar radio structure as a guide to what optically variability might be expected. Similar objects dominated by an extended steep radio spectrum source¹⁰ undergo slow variations with occasional abrupt changes of slope and periods of constancy. This is seen in Fig. 1. The maximum amplitude that might be expected is 1–1.5 mag (unpublished data) which is well in line with that so far observed.

The photometry does allow some constraint to be placed on difference in light travel times. Long delays are excluded by both the POSS and the 1979 plates showing $\Delta m \approx 0.3$. So $\Delta t < 24$ yr. From the photometry in Fig. 1, if A follows B1 then $\Delta t > 1.5$ yr. However, if B1 follows A then $\Delta t > 2$ yr. This will be increased to ~ 3 yr if B1 returns to its previous luminosity at the current rate of decline. In a recent analysis Young *et al.*⁵ gave the time delay $\Delta t < 6$ yr with component A preceding B1. Combining this with the minimum delay from the discussion above gives a delay $2 < \Delta t < 6$ yr. If the sense of the delay is correct then the feature recently observed in the light curve of component B1 has already passed in component A and 2 yr at constant luminosity are still to come for B1. The third image B2 is very faint and its effect on the time delays involving B1 will be negligible⁵.

An alternative cause of luminosity variations may be changes within the gravitational lens^{11–13}. According to Canizares¹⁴ as an individual star passes through the beam from a quasar the luminosity will be increased typically by several magnitudes. The time scale for this variation is ~ 15 yr but depends on the size of the beam, the mass of the star and the velocities involved. In the case of the double quasar Gott¹⁵ considers that very low mass stars will dominate and produce variations in 1–40 yr. On this basis component B1 is expected to be the more variable as it is seen through regions of higher stellar density than component A at ~ 5 kpc from the centre of the galaxy. However, Young¹⁶ considered the effects of very low mass stars to be virtually undetectable and suggested that even with a normal stellar population there will be a sufficient number of stars in the beam for the effects to overlap. This means component B1 should vary continuously by up to 50% over 100 yr while component A undergoes the occasional ~ 15 -yr outburst. According to Gott¹⁵ component B1 should generally be brighter than $I_{B1} = 0.75 I_A$ ($\Delta m \leq 0.3$) while Young¹⁶ predicted substantial variation about this value. Both appear to be at odds with the observations for on only one (1980) of the epochs, 1902, 1903, 1915 (Keel)⁸, 1953, 1955 (POSS), 1979, 1980 on which the double quasar has been observed has the ratio been substantially different from $I_B = 0.75 I_A$ ($\Delta m = 0.3$). With a large beam size the ratio can be kept constant for ~ 50 yr but it is then impossible to duplicate the rapid changes recently observed. Conversely when the type of variations in Fig. 1 can be modelled the long-term variations are at odds with the prediction. Even in favourable conditions Young¹⁶ considered the recent variations to be too large and too

rapid to be due to the passage of stars through the beam. In conclusion the variations in the long and short term are inconsistent with those predicted for the passage of stars through the beam but are consistent with intrinsic variability.

I will make a final comment on the luminosity of the quasars involved in 0957+56AB and the other gravitational lens candidate 1115+080 ABC (ref. 17). If the amplifications of ~ 6 for 0957+56A and ~ 14 for 1115+08A are correct then the luminosities of the unamplified quasar are $M_B \sim -23.6$ and -24.4 respectively ($H = 100 \text{ km s}^{-1} \text{ Mpc}^{-1}$, $q = 1$). These are far in excess of the luminosities of Seyfert galaxies and seriously undermine the suggestion that all quasars are gravitationally lensed Seyfert galaxies¹⁸.

Both components of the double quasar are variable by at least 1.0 mag and show behaviour typical of intrinsically variable quasars with extended radio structure. The near constancy of the difference between the two components at several epochs is contrary to what is expected if the variations are due to the

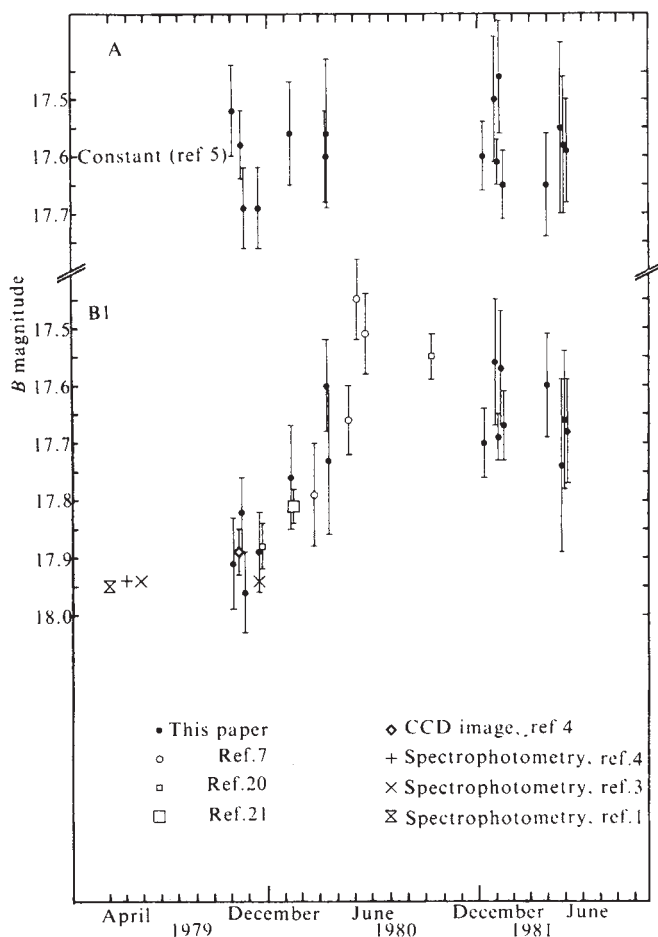


Fig. 1 The light curve of 0957+56A and B1 from April 1979 to June 1981. The diagram contains 16 new measurements of both components together with 12 differential measurements from various authors. For the Herstmonceux photometry unfiltered IIAO emulsions were used which give magnitudes very close to the B band. Both components were reduced independently relative to a sequence of comparison stars. As no photoelectric sequence is available the relative magnitude scale was obtained through reference to two well calibrated fields from the Herstmonceux quasar monitoring programme¹⁹. The error in the relative magnitudes is $\leq 10\%$ and this component is included in the error bars. The zero point of the B magnitude scale is taken as the mean value of component A which is defined as $B = 17.6$ (as given by Young *et al.*⁴) and has an uncertainty of ± 0.1 mag. Differential measurements from photometry and spectrophotometry given by other authors are plotted on the assumption that component A is constant throughout this period. This is supported by Keel⁸ and by Young *et al.*⁵ who find A constant between April 1979 and July 1980.

passage of stars through the beam. Recent photometry places a lower limit of 2 yr on the time delay between the components and if component B1 follows A as calculated by Young *et al.*⁵ then the feature observed in the light curve of B1 has already passed in component A. Analysis of archive plates should reveal more about long-term changes but the question of the time delay should be resolved by continued detailed monitoring.

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First remote sensing of the plasmopause by terrestrial myriametric radiation

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The Earth's plasmopause is a source of natural radio emission in the myriametric wavelength range ($\lambda_{\text{vac}} \sim 10 \text{ km}$) and several mechanisms have been proposed for its generation. One such theory advocates the mode conversion of electrostatic waves to electromagnetic radiation by propagation through a radio window. The window properties are such that the escaping terrestrial myriametric radiation is beamed away from the magnetic equatorial plane at an angle which depends on the plasma parameters at the window. This property should allow myriametric radiation observed by spacecraft to be utilized for remote sensing of the plasmopause. An example derived from the satellite GEOS 1 is presented here in which a radial profile of cold plasma density is obtained for the first time by this technique. The method may also allow a better identification of those electrostatic modes existing in the mixture of hot and cold plasma components at the plasmopause and beyond.

Trapped non-thermal continuum (NTC) is used to describe broad-band radio noise at frequencies $\leq 40 \text{ kHz}$ observed by spacecraft in the plasmatrough, the cavity of low-density plasma between the Earth's plasmasphere and magnetosheath¹. Escaping NTC is noise at higher frequencies which propagates through the magnetosheath into the solar wind². NTC is now widely believed to be produced not as a continuum, but as distinct beams of radiation, with different frequencies emanating from different, albeit possibly adjacent regions of the plasmopause^{2,3}. After multiple reflections at the plasmatrough boundaries the beams from numerous sources are scattered and merge to produce the radio continuum. Myriametric describes the free space wavelength of the radio noise and brings the nomenclature in line with other planetary beamed emissions³.

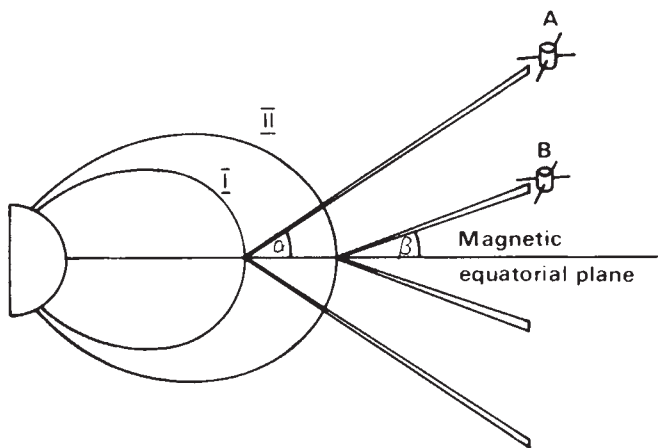


Fig. 1 Representation of the beaming of 30-kHz TMR away from the geomagnetic equatorial plane. The observation of the waves by satellite A allows the position of plasmopause I to be determined. If the observation is made on satellite B, the plasmopause will lie at location II.

The source of terrestrial myriametric radiation (TMR) seems to lie in electrostatic upper-hybrid-resonance (UHR) waves³⁻⁶, which are observed to be most intense within $\pm 1^\circ$ of the geomagnetic Equator at and beyond the plasmopause in the morning sector⁷. Theories of TMR production mechanisms have been recently reviewed by Melrose⁶; the one considered here invokes the propagation of UHR wave energy through a radio window^{3,4}. The window possesses unique properties in that the TMR will be left-hand polarized¹ and beamed away from the geomagnetic equatorial plane at an angle dependent on the ratio f_{ce}/f_p at the window, where f_{ce} and f_{pe} are the electron cyclotron and plasma frequencies respectively³. These properties allow satellite observations of TMR far removed from the source region to be used to determine the radial profile of cold plasma density at the plasmopause. If the satellite later traverses the source region, *in situ* measurements should provide confirmation of the derived profile and information on the relationship between hot and cold plasma components at the plasmopause and beyond.

The term 'window' is used here to denote the phenomena whereby waves in one magnetoionic mode can penetrate through a region where, according to simple ray theory, they would be evanescent, and can emerge on the far side in a different magnetoionic mode (see ref. 8 for a recent review of

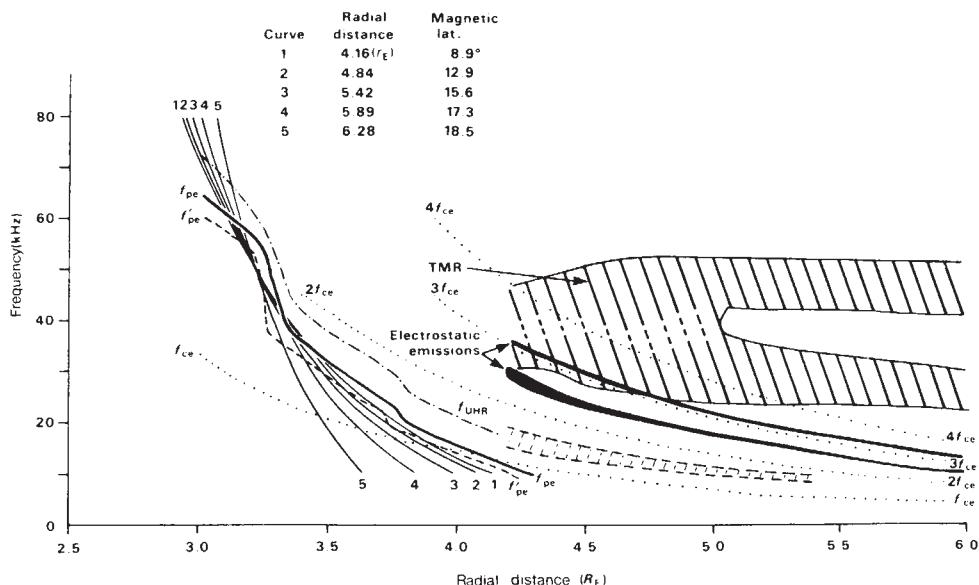
window theory). In the case of propagation of UHR-Z mode waves through a radio window to become left-hand polarized ordinary (L-O) waves, it may be shown that for $f_{ce} < f_{pe}$, the 6-dB window half-widths at the plasmopause for TMR frequencies are typically 1° – 2° . Such narrow beams, together with the restricted latitudinal dimensions of the UHR source region, allow remote satellite observations of TMR to be utilized to determine the radial position of the window. Thus, because the frequency at which TMR exits from the window is f_{pe} , the plasma density N_e , being proportional to f_{pe}^2 , may be determined. Figure 1 illustrates the technique by showing two possible positions, I and II, of the plasmopause. In position I, TMR at 30 kHz is beamed away from the geomagnetic equatorial plane³ at angle $\alpha = \tan^{-1}(f_{ce}/f_{pe})^{1/2}$, whereas if the plasmopause is in position II the same frequency would be beamed at a different angle β since $f_{ce} \propto 1/R_0^2$ has changed, R_0 being the radial distance of the source; the beamwidth in both cases is only 1° – 2° and the beams will have mirror images about the equatorial plane. Satellite A, located at radial distance R and magnetic latitude η , will observe TMR source I, whose radial position R_0 may be determined from the equation:

$$R_0^2 R^2 \sin^2 \eta / f_{ceq} - R_0^2 + 2R_0 R \cos \eta - R^2 \cos^2 \eta = 0 \quad (1)$$

where f_{ceq} is the equatorial gyrofrequency at the Earth's surface. A more general expression exists for the case where the satellite and source are not in the same magnetic meridian plane. Similarly, satellite B observations would allow the source position to be determined when the plasmopause is in position II. Note that trapped NTC need not show any beaming effect with respect to the equatorial plane, because after multiple reflections one would expect the plasmatrough to be filled with NTC. However, escaping NTC which has not suffered reflections is synonymous with TMR and should show beaming².

During the period 0200–0400 UT 4 March 1978, a very stable TMR pattern³ was observed by the satellite GEOS 1 as it moved inbound from $6.2R_E$ to $4.2R_E$. The satellite changed during that time only 5° long, with respect to a plasmopause source corotating with the Earth. From the way in which the signal intensity increased as the plasmopause was approached it is evident that the source and the satellite were close to being in the same meridian plane. The satellite magnetic latitude decreased nearly linearly from 18° to 8° over the above radial distance. Figure 2 shows a sketch of the TMR spectrogram (see ref. 3 for original) which, beyond $5R_E$ exhibits two bands, one in the region of 50 kHz and the other at lower frequencies; the bands merge into one at smaller radial distances. The variation in the upper and lower frequency limits of the lower band is due to the satellite

Fig. 2 Curves 1–5 show TMR sources theoretically visible to GEOS 1 at the five positions shown in the inset table during the inbound leg on 4 March 1978 at ~ 0600 LT. Also shown are the wave emissions seen by the satellite during this period. The hatched region is TMR, whereas the more intense narrow band emissions between gyroharmonics are electrostatic. The weaker emission between f_{ce} and $2f_{ce}$ is linked to the cold upper hybrid frequency f_{UHR} as determined from remote sensing. The 'virtual' and 'real' plasmopause are depicted by f'_{pe} and f_{pe} respectively.



intersecting TMR beams of different frequencies during its inbound traverse³. The table in Fig. 2 shows five satellite locations at which equation (1) was applied assuming, in the first instance, that the whole frequency range 10–80 kHz was visible to GEOS 1. The corresponding source profiles are labelled 1–5 in Fig. 2, and it is immediately apparent that the five curves all intersect close to 50 kHz at $3.22R_E$. The significance of this is that if the plasma frequency is 50 kHz at $3.22R_E$, the satellite should observe that frequency during the whole of its inward traverse from $6.2R_E$ to $4.2R_E$, which indeed it does.

By utilizing the spectrogram and a complete family of theoretical curves similar to 1–5 in Fig. 2, one can determine the source positions at other frequencies and hence obtain a radial profile of plasma density. For example, if at a given frequency and satellite position no TMR is observed, this indicates that the source does not lie on the corresponding theoretical profile, whereas if TMR is visible the source position for that frequency can be determined from the relevant curve. The radial plasma frequency profile constructed in this way for 4 March 1978 is labelled f_{pe} in Fig. 2. A small correction to allow for wave refraction at the window has to be applied to this profile as equation (1) was derived assuming linear propagation. Ray tracing calculations indicate that $\sim 0.08R_E$ has to be added to the radial positions of the sources and thus the 'true' plasma frequency profile is shown labelled f_{pe} .

Figure 2 shows (dotted) the electron cyclotron frequency f_{ce} and its harmonics. The strongest natural emission band observed in the spectrogram lies between $2f_{ce}$ and $3f_{ce}$, whereas the much weaker emission between f_{ce} and $2f_{ce}$ is the one that links smoothly to the f_{UHR} profile as calculated from $f_{UHR} = (f_{pe}^2 + f_{ce}^2)^{1/2}$. Note that GEOS 1 was at magnetic latitudes greater than 8° when the spectrogram was recorded. Unfortunately, the satellite was subject to its end of orbit switch-off before it traversed the predicted source region and hence no direct *in situ* observations could be made on that particular pass. However, on the previous day, GEOS 1 remained in operation as the equatorial plane was crossed and the TMR observations, although themselves more limited in extent and more complicated in spectrum than on 4 March indicated that the plasmopause position was in approximately the same position. As for 4 March 1978, the strongest electrostatic emissions lay between $2f_{ce}$ and $3f_{ce}$ when the satellite was at magnetic latitudes $\geq 3^\circ$, but as the Equator was approached the weak emission between f_{ce} and $2f_{ce}$ increased in intensity and became the most intense at the Equator. Therefore, from the indirect evidence on 4 March and the more direct evidence on 3 March, the cold UHR emission apparently dominates only very close to the Equator, whereas the stronger emission at other latitudes is at a higher frequency. A detailed study of this effect and of the possible extension of this technique to separate hot and cold plasma components will be reported separately.

The main conclusions, therefore, are that the unique properties of the radio window from which TMR appears to emanate can be used for remote sensing of the plasmopause. When the technique is applied to certain GEOS 1 data, the results indicate that the stronger non-equatorial electrostatic emissions are at frequencies higher than the cold UHR frequency. Emissions at the latter frequency dominate only within $\sim \pm 1^\circ$ of the geomagnetic Equator, and their identification may allow the separation of hot and cold plasma populations. As similar emissions to TMR and to NTC have been observed by the Voyager spacecraft at Jupiter and Saturn, these results could have considerable significance for the understanding of the environments of other planets, and could provide a diagnostic tool to study regions of other planetary magnetospheres not accessible to *in situ* observations by spacecraft.

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Atmospheric angular momentum and the length of day: a common fluctuation with a period near 50 days

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Semiannual, annual and some longer period changes in the rotation of the Earth have long been attributed to meteorological causes^{1–3}. More recently some higher frequency variations in the length of day (l.o.d.) were shown to be strongly correlated with variations of the polar component of the angular momentum of the atmosphere^{4,5}. However, fluctuations in l.o.d. with periods near 50 days were not definitely identified, because either the sampling was too coarse⁴ or it extended over too short an interval⁵. Fluctuations in tropical wind speeds with periods in this range have, nevertheless, been studied^{4,6,7}, but their counterparts, although expected⁴, had not been identified in the Earth's rotation. Last year, four astronomical measures of changes in l.o.d. obtained in 1979 were shown to exhibit nearly the same ~ 50 -day fluctuation⁸. To find out whether this fluctuation was persistent and of meteorological origin we analysed lunar laser ranging (LLR) observations from the McDonald Observatory in Texas and zonal (east–west) wind data deduced from sources distributed over the globe. The relevant data were available for the common period 1976–79. We describe these analyses in turn and then compare the results.

Values of l.o.d. were obtained by smoothing and then differentiating our LLR estimates⁹ of the rotation of the Earth as a function of time. These values were corrected for the effects of polar motion with pole positions determined by the Bureau International de l'Heure¹⁰. Our results were tabulated at 5-day intervals from 2 January 1976 to 27 December 1979. Typically each tabulated value of l.o.d. has an estimated standard error of ~ 0.1 ms. The uncertainties, however, vary over the 4-yr interval, due in part to the uneven distribution in time of the LLR observations.

To investigate the short-term variations in l.o.d., we used an *ad hoc* procedure that seems adequate for the purpose. First we divided the l.o.d. time series into nearly year-long segments. For each segment we determined the mean level and, using least squares analysis, the best-fitting Fourier components with annual and semiannual periods. We then removed these components, as well as the fortnightly and monthly tidal terms, from the l.o.d. values. The residual variations are shown for each year in Fig. 1a–d. Each curve is dominated by fluctuations with periods in the range 40–60 days. The typical amplitude of these fluctuations is ~ 0.2 ms, or about half the mean amplitudes of the annual and semiannual terms; the lack of continuity between the curves is due to the procedure used to remove the low-frequency components of the l.o.d. variations.

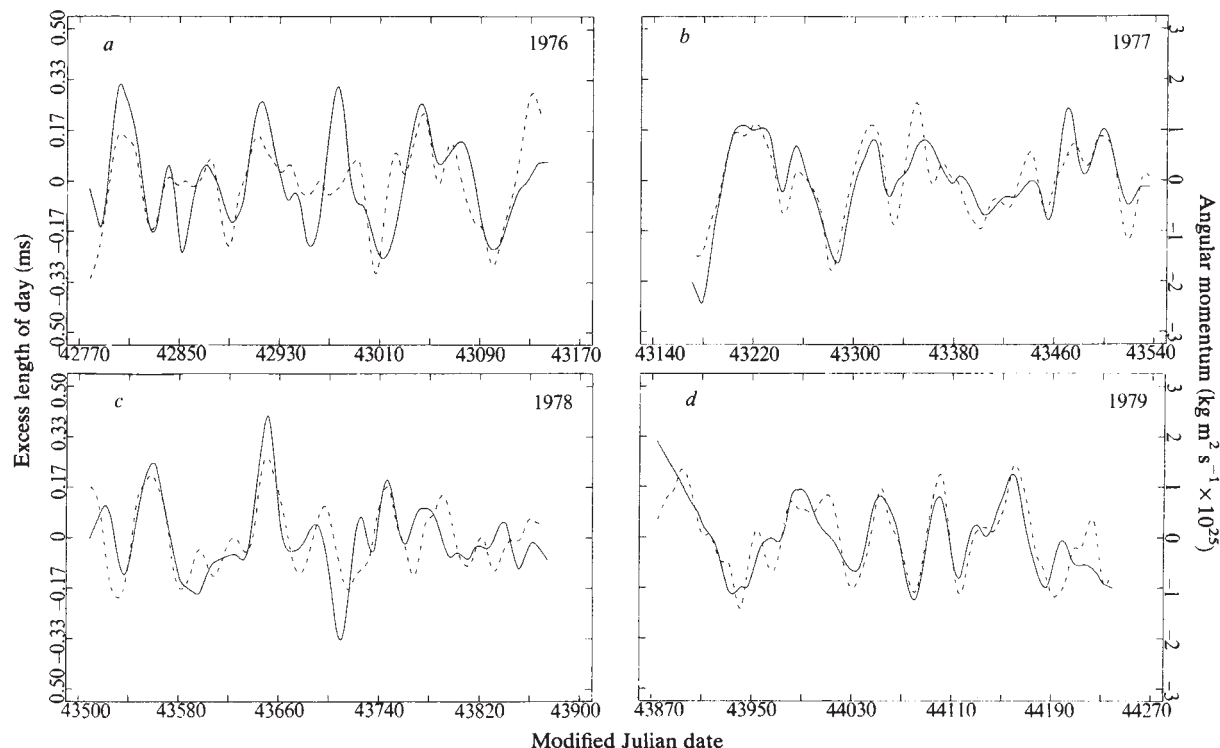


Fig. 1 Variation in length of day determined from lunar laser ranging observations (solid line) and from the angular momentum of the atmosphere (dashed line) for the years 1976–79. The annual and semiannual variations and mean levels of both sets of l.o.d. values, as well as the fortnightly and monthly tidal terms in the lunar laser ranging values, have been removed (see text).

Although appropriate data on the angular momentum of the atmosphere are not available for the time before 1976, useful lunar laser ranging observations extend back to 1971. These, too, exhibit significant fluctuations with periods in the range 40–60 days. Power spectra of the entire set of l.o.d. values, and subsets of them, will be presented and discussed elsewhere.

Our time series of values of the angular momentum of the atmosphere was derived from analyses of the global distribution of zonal winds made twice daily (for 00.00 and 12.00 UTC) by the US National Meteorological Center (NMC)¹¹. These analyses are based on data gathered from various sources, including rawinsonde balloons, satellites and commercial aircraft, and provide wind velocities over a grid with points spaced every 2.5° in both latitude and longitude, at each of 12 pressure levels ranging from 1,000 to 50 mbar. (A detailed analysis of the spatial and temporal variability of these zonal winds will be presented elsewhere¹².) The values of wind velocity at the grid points were zonally averaged and then, with appropriate weighting, integrated over latitude (ϕ) and pressure (p) to determine, relative to an Earth-fixed frame, the approximate angular momentum of the atmosphere about the polar axis:

$$h_3(t) = \frac{2\pi a^3}{g} \int_{100 \text{ mbar}}^{1,000 \text{ mbar}} \int_{-\pi/2}^{\pi/2} [u] \cos^2 \phi \, d\phi \, dp \quad (1)$$

where $[u]$ is the zonal average of the eastward component of wind velocity, a is the mean radius of the Earth and g is the acceleration due to gravity. Equation (1) is based on the assumption that the atmosphere is in hydrostatic equilibrium and ignores the variation with altitude and latitude of the distance of a parcel of air from the centre of the Earth. Because of the expected unreliability of the data at pressures below 100 mbar (ref. 13), we have ignored these data. Test calculations, using the low pressure data, indicate that deleting them incurs a systematic error of ~10% in the determination of the mean value of h_3 , but we expect this deletion to have an even smaller impact on day-to-day changes in h_3 . Of greater effect on these changes are the errors that result from gaps in the spatial

distribution of observations, inaccuracies in the basic wind measurements and approximations in the NMC's analysis. We cannot place a reliable quantitative limit on these errors; however, model calculations related to the first two error sources¹⁴ and comparisons related to the third⁵ indicate that these errors *in toto* are typically ~10% or less.

We determined h_3 from equation (1) for twice-daily epochs daily extending from 1 January 1976 to 31 December 1979. The results from December 1978 to November 1979 are thought to be the most accurate because of the intense effort devoted to data collection under the Global Weather Experiment¹⁵.

Fluctuations in h_3 on time scales of the order of 1 yr or less probably affect only the crust and mantle (hereafter 'shell') of the Earth because the core is thought to participate only in longer period variations^{5,16}. For variations with periods <1 yr, then, the relation between the changes Δh_3 in h_3 and the corresponding changes $\Delta\omega$ in the angular velocity of the shell of the Earth is given by

$$\Delta\omega \approx -\frac{\Delta h_3}{I_s} \quad (2)$$

where I_s is the principal (axial) moment of inertia of the shell. A change in the angular velocity of the shell is related to a change in the observed l.o.d. by

$$\frac{\Delta \text{l.o.d.}}{\text{l.o.d.}} = -\frac{\Delta\omega}{\omega} \quad (3)$$

Combining equations (2) and (3) and using the following constants: $I_s \approx 7.04 \times 10^{37} \text{ kg m}^2$ (this value of I_s was derived from the B1 density model in ref. 17; the uncertainty in I_s is probably not greater than 2%), $\omega = 7.29 \times 10^{-5} \text{ s}^{-1}$, l.o.d. $\equiv 86,400 \text{ s}$, yields

$$\Delta \text{l.o.d. (s)} = 1.68 \times 10^{-29} \Delta h_3 (\text{kg m}^2 \text{ s}^{-1}) \quad (4)$$

The mean level of h_3 between 1 January 1976 and 31 December 1979 is $\sim 1.4 \times 10^{26} \text{ kg m}^2 \text{ s}^{-1}$. This amount of angular momentum transferred to the solid Earth (excluding its core) would change the l.o.d. by 2.3 ms.

As with the values derived from LLR observations, we removed from the l.o.d. values obtained from equations (1) to (4) the mean level and the best-fitting Fourier components with annual and semiannual periods for each yr-long segment separately. The residuals were smoothed using a gaussian-shaped low-pass filter, with a window width in the time domain of about 8 days (full-width-at-half-maximum). This degree of smoothing is consistent with the smoothing applied to the LLR estimates of Earth rotation. Figure 1a-d shows the smoothed curves for each year superimposed on the l.o.d. curves obtained from the lunar laser ranging observations. In common with the LLR curves, those derived from the estimates of h_3 are discontinuous from year to year. The scale on the right-hand side is related to the scale on the left through equation (4).

The degree of correlation between the observed (LLR) and the inferred (meteorological) values of the changes in l.o.d. is quite striking; in most places there is good agreement in both the amplitude and phase of the variations. The correlation seems highest in 1979, perhaps due at least in part to the special attention devoted in that year to the collection of meteorological data. Overall, the comparison in Fig. 1 implies strongly that these ~50-day period fluctuations in l.o.d. are 'real' and are related to meteorological effects. The uncertainties in the LLR l.o.d. values and in the estimates of h_3 are large enough to account for the differences in the two sets of curves and therefore too large to make a detailed analysis of these differences worthwhile. These uncertainties are also too large to permit us to place useful limits on the magnitudes of any non-atmospheric contributions to the changes in l.o.d. Thus, we cannot determine from the comparison whether the core participates in these l.o.d. changes nor can we place useful constraints on the contributions of ocean currents and other neglected torques. Other studies¹⁶ imply that the contributions of ocean currents are small. Our calculations indicate that the contributions of other neglected torques are also small.

The exchange of momentum between the Earth's atmosphere and surface has a central role in determining both the structure of atmospheric flow and the dynamics of the Earth's rotation. Observed changes in l.o.d. can provide a constraint for models of atmospheric flow and a (partial) check for global analyses of such motions. The removal of more accurate estimates of the atmospheric contribution to changes in l.o.d. observations might reveal residual variations associated with ocean currents, earthquake fault displacements and core-mantle interactions.

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Solvated electrons in the upper atmosphere

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Solvated electrons have been postulated¹⁻³ to exist in liquids and solids, and recent laboratory measurements⁴ have revealed that they exist even in the gas phase. In particular, these measurements suggest that eight water molecules are sufficient to bind an electron and give rise to a solvated electron of the kind $(\text{H}_2\text{O})_8^-$. This seems to be consistent with theoretical considerations⁵ suggesting that a dipole of molecular size must have a minimum dipole moment of about 2.5 D to support a bound state. According to theoretical calculations⁶, a cold, neutral water cluster $(\text{H}_2\text{O})_8$ has a dipole moment of 4.61 D, so that larger $(\text{H}_2\text{O})_n$ clusters may be expected to bind an electron. Building on these ideas I consider here the possibility that solvated electrons may also exist in the Earth's upper atmosphere and that they may affect nucleation and aerosol formation. Furthermore, I suggest that upper-atmosphere solvated electrons may be detected, using rocket-borne ion mass spectrometers, and that they may be used as tracers for probing condensation nuclei.

In the Earth's upper atmosphere solvated electrons may be formed by attachment of free electrons to clusters of molecules having large dipole moments or polarizabilities. Thus, solvated electron formation would be restricted to regions where both free electrons and the corresponding neutral species are present.

In the atmosphere free electrons essentially exist only at heights above ~60-65 km (day) and 75-80 km (night), whereas negative ions represent the atmospheric negatively charged constituents below these heights⁷. The main reason for the rather abrupt transition from a negative ion to an electron regime is electron detachment induced by oxygen atoms⁸ which become abundant above these heights⁷.

Collisions of free electrons with these molecular clusters may give rise to solvated electrons.

The pHs should, in most conditions, not be large enough to give rise to solvated electrons. Sufficiently large pHs and $(\text{H}_2\text{O})_n$ clusters probably occur only in conditions of extremely low temperatures (<150 K) around the mesopause⁹. Such conditions are occasionally met at high geographical latitudes during local summer. In fact, large pHs containing up to 20 water molecules were recently detected by Bjorn and Arnold¹⁰ using a rocket-borne ion mass spectrometer.

It has been suggested previously^{9,11} that pHs may induce the nucleation of atmospheric water vapour in the cold mesopause region and thereby give rise to a mesospheric haze layer and to noctilucent clouds. As pointed out elsewhere⁹, however, ion nucleation, although being thermodynamically possible, may suffer from severe kinetic limitations. pHs may be rapidly destroyed by dissociative electron recombination and therefore not grow to critical sizes.

Here, I propose an alternative recombination mechanism involving solvated electron formation. Instead of neutralizing the excess proton contained in a large pH the electron may be solvated by water molecules attached to the proton. Thus, a charge doublet composed of a solvated electron and a solvated proton may be formed. Taking measured enthalpy differences for successive proton hydration¹² it seems that 11 water molecules are sufficient to stabilize a pH against spontaneous neutralization of its excess proton. In fact, this number of H_2O molecules is already larger than the eight required for electron solvation.

A doublet may grow by attachment of water molecules and ultimately pick up another electron and thereby be converted to a triplet containing a solvated proton and two solvated electrons. The formation of positive triplets is much less likely as the rate of positive ion collisions is much less than that of electron collisions due to the about 100,000 times larger ion mass. As the triplet carries a negative net charge, it should readily recombine with a positive ion, generally a pH giving rise to the formation of a quasineutral quadruplet. Further electron and ion attachment may lead to larger multiplets. These resemble the stratospheric polyions or multi-ion complexes I recently discussed¹³ which, however, contain negative ions rather than solvated electrons.

If the charge multiplets were in fact formed at the mesopause, they may have an important bearing on nucleation, as they may stabilize subcritical water cluster against evaporation and allow for the build-up of critical clusters by electron, pH , and H_2O attachment.

I now attempt to estimate the abundances of doublets and quadruplets at the mesopause for a steady state situation.

Assuming that doublets are formed by electron recombination of large pH s (fractional abundance f) at a rate coefficient α and are lost only by electron attachment (rate coefficient k), the following steady-state continuity equation is obtained

$$f[pH]\alpha_e[e] = [D]k[e] \quad (2)$$

which relates the concentration of doublets $[D]$ to the pH and total electron concentrations. Taking $\alpha_e = 10^{-5} \text{ cm}^3 \text{ s}^{-1}$ (ref. 14) and assuming that every collision of an electron and a doublet leads to electron attachment ($k \approx 10^{-7} \text{ cm}^3 \text{ s}^{-1}$) we obtain

$$[D] \approx f10^2[pH] \quad (3)$$

Analogously we obtain the steady-state continuity equation for triplets

$$[D]k[e] = [T]\alpha_1[n_+] \quad (4)$$

where α_1 is an ion-ion recombination coefficient (usually^{15,16} $\sim 6 \times 10^{-8} \text{ cm}^3 \text{ s}^{-1}$) and $[n_+]$ is the total positive ion concentration. Assuming $[n_+] = [pH] = [e]$ we obtain

$$[T] \approx [D] \quad (5)$$

Evidently the above estimates can, if at all, only apply for f smaller than 0.01, because otherwise triplets would become more abundant than electrons. This would give rise to a depletion of electrons by triplet formation which is in conflict with the assumption of $[e] = [n_+]$. Hence, if f was > 0.01 , one should expect that some depletion of electrons actually occurs, the rates for doublet and triplet formation, however, decrease.

Note that a decrease of $[e]$ due to electron solvation would increase the lifetime of smaller pH s, which are destroyed by dissociative electron recombination, and thus would increase the rate of formation of large pH s. Thus, there would be a strong positive feedback in ion nucleation.

Electron solvation may, of course, be induced by $(H_2O)_n$ clusters also if these are not formed by ion nucleation but by some other process, as for example, homogeneous nucleation of water vapour condensation on pre-existing particles.

Having discussed electron solvation by neutral and positively charged water clusters, I now consider electron solvation by 'meteoric smoke particles'. These molecular clusters may contain compounds with large dipole moments and polarizabilities which enable them to solvate an electron strongly, because the presence of meteor smoke particles is probably not limited in space and time electron solvations by these clusters may be a common process around the mesopause.

Taking surface area densities for meteoric smoke particles predicted by the model of Hunten *et al.*¹⁷ and assuming that solvated electrons are lost by recombination with positive ions, an upper limit to the abundance ratio for solvated electrons and electrons of about 0.1 is estimated.

Finally, if solvated electrons were indeed as abundant as estimated above, they should be easily detectable in the meso-

pause region using rocket-borne mass spectrometers. *In situ* mass spectrometric detection of solvated electrons may be a powerful tool of probing molecular clusters such as $(H_2O)_n$ and meteoric smoke particles in the mesopause region.

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Halocarbons in the stratosphere

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The possible impact of chlorine compounds on the Earth's ozone layer has caused concern. Profiles of the anthropogenic halocarbons F-11 ($CFCl_3$) and F-12 (CF_2Cl_2) have already been measured in the stratosphere¹⁻⁴. Measurements of the vertical distribution of methyl chloride (CH_3Cl), the most important natural chlorine-bearing species confirm that chlorine of anthropogenic origin now predominates the stratosphere^{5,6}. More halogen radicals are added through decomposition of various other halocarbons, most of them released by man. We report here the first measurements of vertical profiles of F-13 (CF_3Cl), F-14 (CF_4), F-113 ($C_2F_3Cl_3$), F-114 ($C_2F_4Cl_2$), F-115 (C_2F_5Cl), F-116 (C_2F_6), and F-13 B (CF_3Br) resulting from gas chromatography-mass spectrometer (GC-MS) analysis of air samples collected cryogenically between 10 and 33 km, at 44° N. Some data for F-22 (CHF_2Cl), methyl bromide (CH_3Br) and methyl chloroform (CH_3CCl_3) also presented are subject to confirmation.

Cryogenic sampling with subsequent analysis of the samples in the laboratory is an excellent tool for simultaneously measuring a variety of stable trace gases in the atmosphere^{1,2}; vertical profiles of H_2 , CH_4 , CO , CO_2 , N_2O , $CFCl_3$, CF_2Cl_2 and CH_3Cl have already been measured up to ~ 36 km altitude. From a recent balloon flight of the MPAE neon-cooled sampler launched on 25 September 1980, in southern France (44° N), seven stratospheric air samples were recovered. While the first one of these had been taken during the ascent at 10 km height, the six other samples were collected during the descent of the helium-filled balloon at 33.2, 28.8, 25.9, 23.3, 20.0 and 14.4 km, respectively. These figures correspond to the centres of the sampling height intervals whose ranges varied between 0.2 and 0.5 km. The slow descent, at a rate of ~ 50 – 100 m min^{-1} , was achieved by releasing gas through a remotely controlled valve on top of the balloon.

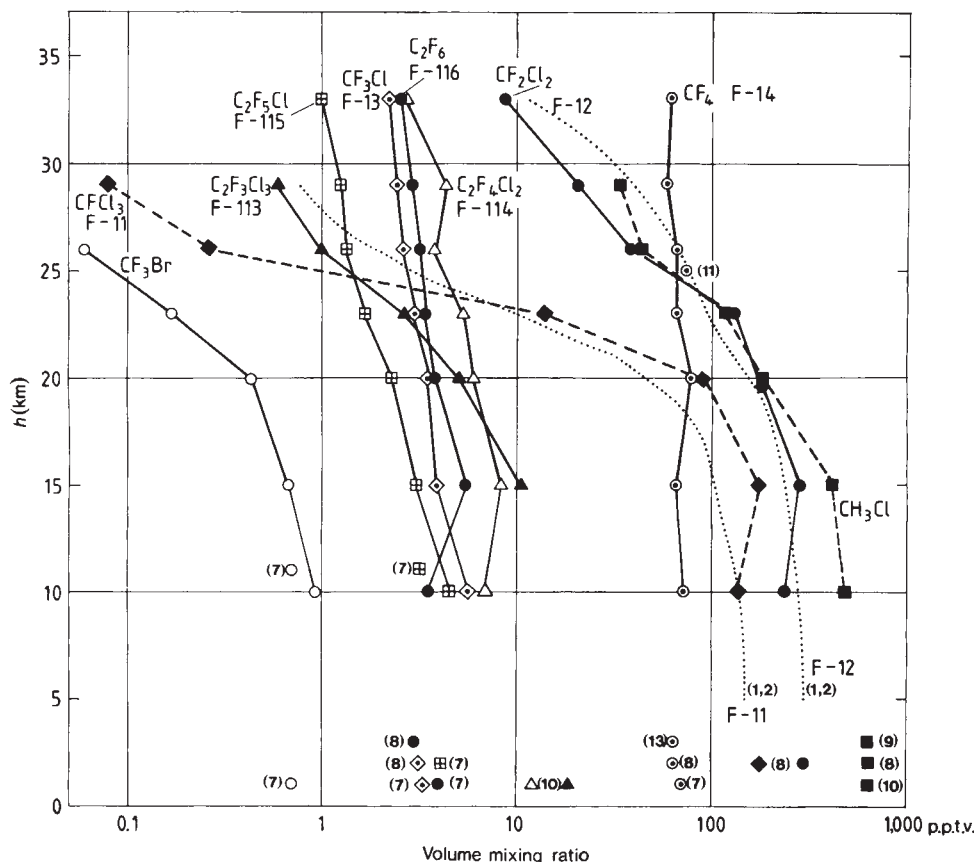


Fig. 1 Vertical profiles of halo-carbons measured at 44° N by GC-MS analysis of cryogenically collected balloon samples. Results from measurements made by other authors (reference numbers in parentheses are shown for comparison). The tropospheric data shown at the bottom are not related to the height scale.

Immediately after the flight, the samples were transferred into 1.6-l stainless steel bottles electropolished internally and fitted with metal bellows valves. Each bottle contained a sample of about 8–10 l of air at STP that was analysed three weeks later using a GC-MS combination at AERE Harwell (Hewlett Packard 5710 GC/VG Micromass 16 FMS). As repetitive analyses of previously collected samples had shown, F-13, F-14, F-113, F-114, F-115, F-116, and F-13B remain stable in these bottles over a period of more than 6 months (S.A.P. and R. A. Rasmussen, unpublished data). Because many of the concentrations to be measured were below the detection limit for direct analysis of a 5-ml sample, a cryotrap concentrator described elsewhere⁷ was used for all analyses. The volumes of air used for each estimation varied between 100 ml and 1 l, which is a substantial fraction of the total sample. Duplicate analyses were generally not performed so that data on a wide range of species could be recorded. The precision of the analysis is $\pm 15\%$ for every individual data point, while the error of the absolute calibration is $\pm 10\%$ for all constituents shown here. Typically the detection limit is of the order of 0.1 p.p.t.v. (1 p.p.t.v. = 1 part in 10^{12} by volume) for a 1-l sample.

The results are plotted in Fig. 1. The upper parts of some of the profiles, where the mixing ratio fell below the detection limit, are not shown. Measurements made by other authors are included for comparison.

The low-altitude sample collected during the ascent at 10 km was obviously diluted by the helium that had been filled into the air intake system of the cryogenic sampler before launch. This is confirmed by the comparison of the data from the 10-km balloon sample with those obtained from samples collected cryogenically aboard a Learjet aircraft over southwestern UK at almost the same time and altitude (S.A.P. and R. A. Rasmussen, unpublished data). For most constituents, the mixing ratios analysed from the balloon sample taken at 10 km are lower than those obtained from the Learjet samples. A correction factor can be applied to this lowest balloon sample based on measurements of common constituents such as F-11 and F-12 in both balloon and aircraft samples. This suggests that the mixing

ratios in the 10-km balloon sample are $\sim 20\%$ low, which needs to be born in mind when referring to the profiles shown in Fig. 1.

Stratospheric profiles of CFCl_3 , CF_2Cl_2 and CH_3Cl have already been documented^{1–6}. Below ~ 20 km the mixing ratios of both CFCl_3 and CF_2Cl_2 are higher than those given by the average profiles measured in June 1979 at the same latitude^{1,2}, also shown in Fig. 1 (dotted lines). Between June 1977 and June 1979, an increase of the average tropospheric mixing ratio of CFCl_3 from 130 to 147 p.p.t.v. had been observed at 44° N. During the same time CF_2Cl_2 increased from 240 to 292 p.p.t.v. (refs 1, 2). The increase between June 1979 and September 1980 shown here is in reasonable agreement with that observed earlier. Above 25 km the mixing ratio of F-11 falls off more rapidly than the average profile for 1979.

The stratospheric profile obtained for CH_3Cl agrees fairly well with that measured in June 1978⁵ if the uppermost point is omitted. The increase of the CH_3Cl mixing ratio above 30 km may be due to contamination of the sample collected at ceiling altitude. Three near-surface measurements of 630 p.p.t.v. (ref. 8), 624 p.p.t.v. (ref. 9) and 611 p.p.t.v. (ref. 10) are shown at the bottom of Fig. 1 for comparison. Their display, which was made for the sake of clarity, has no relation to the height scale of Fig. 1. The same holds for the other tropospheric data plotted in Fig. 1. CH_3Cl is a natural constituent with the ocean being its main source⁹.

The other halocarbons whose vertical distributions are shown in Fig. 1 have, to our knowledge, not yet been measured in the stratosphere, with the exception of F-14 (CF_4) for which one data point—75 p.p.t.v. at 25 km—was measured by Goldman *et al.*¹¹. In the troposphere some measurements exist for most of these constituents, and these data have been included at the bottom of Fig. 1 to be identified by the same symbols as used in the stratospheric profiles. Two data points for CF_3Br and $\text{C}_2\text{F}_5\text{Cl}$, respectively, as analysed from aircraft samples taken at 11 km (ref. 7) are also shown for comparison.

F-13 B (CF_3Br), a bromine compound of anthropogenic origin, is used as a fire extinguisher. Its abundance, about 0.7 p.p.t.v. in the troposphere⁷, is mostly due to anthropogenic

release. In the stratosphere CF_3Br mixing ratios decrease, from about 1 p.p.t.v. at 10 km to 0.06 p.p.t.v. at 25.9 km. It has been argued⁷ that this decrease may be due to photolysis by which bromine is released augmenting the effect that natural bromine has on removing ozone. Methyl bromide (CH_3Br) is, like methyl chloride, predominantly of a natural origin. Tropospheric measurements of CH_3Br suggest mixing ratios ranging between 5 and 25 p.p.t.v. (ref. 8). If our measurements are correct, then in the stratosphere the mixing ratio of CH_3Br falls off rapidly: 1.2 p.p.t.v. was measured at 14.4 km, while the sample taken at 20 km was already below the detection limit. These data are not shown in Fig. 1.

The aluminium industry⁷ is probably the major source for both F-14 (CF_4) and F-116 (C_2F_6). F-14 is a very stable constituent¹² showing almost no vertical gradient in the stratosphere. Its mixing ratio was measured as 65 p.p.t.v. at 14.4 km and 62 p.p.t.v. at 33.2 km; the single data point measured by Goldman *et al.*¹¹ fits this profile quite well. Tropospheric CF_4 mixing ratios, with averages of 69.9 p.p.t.v. (ref. 7), 63.6 p.p.t.v. (ref. 13) and 65 p.p.t.v. (ref. 8) measured in the Northern Hemisphere, match our stratospheric data within the quoted error limits. F-116 decreases from about 4 p.p.t.v. measured at 10 km to 2.5 p.p.t.v. at 33.2 km. This difference from F-14 may be due to a more rapid buildup in the atmosphere or due to stratospheric decomposition. The tropospheric mixing ratio of 4 p.p.t.v. measured⁷ in 1979 is in good agreement with our data.

The results for F-13 (CF_3Cl) and F-115 ($\text{C}_2\text{F}_5\text{Cl}$) are interesting. It was suggested earlier that F-115 is growing more rapidly than other anthropogenic species containing the CF_3 grouping, possibly because it is used in conjunction with F-22 (CHF_2Cl) in the refrigerant F-502. This may account for the steeper profile for F-115 whose mixing ratios decrease from 3.1 p.p.t.v. at 14.4 km to 1.0 p.p.t.v. at 33.2 km, compared with that for F-13 which falls from 3.9 p.p.t.v. at 14.4 km to 2.3 p.p.t.v. at 33.2 km. A comparison with tropospheric F-13 mixing ratios, of 3.4 p.p.t.v. (ref. 7) and 3.0 p.p.t.v. (ref. 8) measured in 1979, seems to suggest that F-13 is also increasing with time. For F-115 a tropospheric mixing ratio of 4.1 p.p.t.v. was measured in 1980 (ref. 7).

Our stratospheric profile for F-22 shows a decrease from ~50 p.p.t.v. at 10 km to ~10 p.p.t.v. at 33.2 km. This is an important species to measure both because of its rapid growth rate¹⁴ and because it contains a hydrogen atom and therefore will react with hydroxyl radicals in the stratosphere. Our data must be considered preliminary though, because of inconsistencies apparent on reanalysis after a 3 months gap. The profile is therefore not shown on Fig. 1.

F-113 ($\text{C}_2\text{F}_3\text{Cl}_3$) and F-114 ($\text{C}_2\text{F}_4\text{Cl}_2$) are used as refrigerants. Their tropospheric abundances, which have been measured extensively by Singh *et al.*¹⁰, are 19 and 12 p.p.t.v., respectively, on the Northern Hemisphere. In the stratosphere F-113 is rapidly decomposed, its mixing ratio decreases to 0.6 p.p.t.v. at 28.8 km. The stratospheric decomposition of F-114 is much slower; its mixing ratios decrease from about 8.5 p.p.t.v. at 14.4 km to 2.7 p.p.t.v. at 33.2 km.

Methyl chloroform (CH_3CCl_3), mainly used as a dry cleaning solvent, has been suggested¹⁰ as a potential depleter of stratospheric ozone due to its rapid growth in the lower atmosphere (15.5 p.p.t.v. yr^{-1}). For the Northern Hemisphere Singh *et al.*¹⁰ published an average mixing ratio of 113 p.p.t.v., based on measurements made at the end of 1977, while Rasmussen and Khalil⁸ measured an average of 135 p.p.t.v. during mid-1979. Our vertical profile measured in the stratosphere indicates that CH_3CCl_3 is rapidly decomposed. The mixing ratios decrease from ~100 p.p.t.v. at 10 km to 1 p.p.t.v. at 23.3 km. These measurements were made 3 weeks after the samples had been taken. Analyses taken 3 months later showed that methyl chloroform had completely disappeared from the containers (carbon tetrachloride had already disappeared 3 weeks after sampling, when the first analyses were made; thus no CCl_4 profile can be presented). Because the above values for CH_3CCl_3 are preliminary they are not displayed in Fig. 1. As the

present annual growth rate of CH_3CCl_3 is much faster than that of CFCl_3 (ref. 10) it might add comparable amounts of ClO_x radicals to the stratosphere within a foreseeable time.

Our results demonstrate that besides F-11, F-12 and CH_3Cl there are quite a few halocarbons, mostly anthropogenic, that contribute to the stratospheric halogen budget. Above 30 km the amount of organically bound chlorine present as F-113, F-115, F-13, F-114, and F-22 combined is comparable with that present as F-11 and F-12.

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Anomalous wind estimates from the Seasat scatterometer

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The Seasat-A Satellite Scatterometer (SASS) measured the radar backscatter intensity from the sea surface using a four-beam microwave antenna¹⁻³. Estimates of wind speed and direction derived from these data agree well with surface measurements made during the Joint Air-Sea Interaction (JASIN) experiment⁴⁻⁶, but there are occasions (3 out of 23 satellite passes) when the results are anomalous. We have now studied one such occasion when the satellite measurements differed substantially from those at the surface of the sea and conclude that the interpretation of the SASS measurements may have been vitiated by a mid-level convective system deep enough to produce thunderstorms and lightning.

Figure 1 shows a plot of the SASS wind vectors for Rev. 557 (23.16 GMT, 4 August 1978) together with wind speeds measured by ships at the corners of the JASIN meteorological triangle. Over most of the area a SASS winds are 7 m s⁻¹ or less but in a region centred on 59.1°N and 12.0°W, the SASS speeds are about 17 m s⁻¹, which, given the rather weak pressure gradient, seems unlikely. Moreover, examination of a similar SASS plot for Rev. 556, 100 min earlier, revealed no apparent irregularities at the same location.

To understand this discrepancy we examined the individual normalized radar backscatter coefficients, σ^0 —the basic scatterometer data from which wind vectors are derived. The region of anomalously high winds exhibited localized, large σ^0 obtained

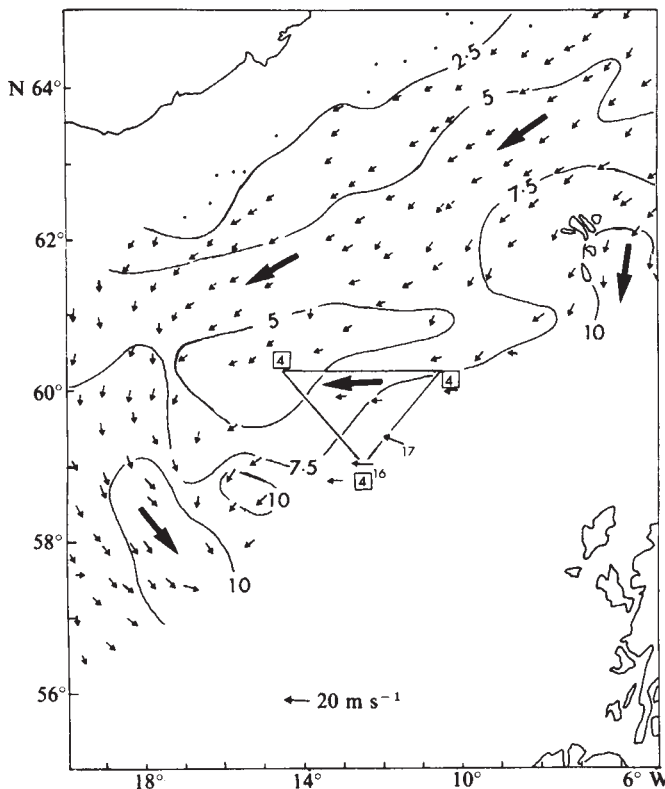


Fig. 1 Scatterometer wind vectors for Rev. 557, 23.16 GMT on 4 August 1978 (wind speeds in m s^{-1}). The harmonic nature of the algorithm used to convert backscatter intensity to wind velocity means that up to four solutions are obtained for each footprint¹⁰, each having similar speeds but differing widely in direction. A preferred direction has been manually selected in each case to ensure dynamic consistency and to give closest agreement with routinely produced surface pressure fields. Anomalous speeds near the southern corner of the JASIN meteorological triangle have been annotated and points without arrows indicate wind speeds of 3 m s^{-1} or less. Numbers in boxes denote speeds measured by ships at the corners of the triangle.

from the backward and forward looking antennas (beams 3 and 4 respectively) on the left-hand side of the satellite for both vertical and horizontal polarizations. Figure 2 shows contour plots of σ° , adjusted to remove incidence angle dependence, for beam 3 vertical polarization. High-backscatter regions along a 300–400 km line lying to the south-east of the JASIN meteorological triangle and aligned NE/SW were not present on the previous pass although near $59^\circ\text{N } 10.5^\circ\text{W}$ there was a suggestion of increased σ° . This corresponded closely with a region of increased liquid water as inferred from the Scanning Multichannel Microwave Radiometer (SMMR) (Fig. 3) and also with a small feature in the synthetic aperture radar image apparently oriented ESE/WNW. Unfortunately, the sampling characteristics of these two instruments meant no information in the area of interest could be obtained on Rev. 557. As these results suggested that the scatterometer anomalies were associated with a meteorological event of rather small scale, the meteorological data available for the JASIN area were studied.

The UK Meteorological Office analysis at 18.00 GMT showed fronts approaching the area from both the west (front A) and from the north-east (front B). Time series of surface winds from several platforms showed that at the passage of B the wind veered by 50° and increased by $3\text{--}4 \text{ m s}^{-1}$. The times at which this change occurred at each of the platforms suggested south-westward propagation of the front at 5 m s^{-1} . A NOAA-5 IR satellite image at 18.16 GMT revealed that B was not accompanied by a well-defined band of cloud. However, at $58.5^\circ\text{N}, 9.5^\circ\text{W}$ there was evidence of deep convective cloud. Subsequent cloud pictures at 20.09 and 21.33 GMT from the Seasat visible

and IR radiometer (VIRR) indicated that the feature moved westwards at $\sim 7 \text{ m s}^{-1}$ to occupy the area $58\text{--}59^\circ\text{N}$ and $10\text{--}12^\circ\text{W}$ at 21.33 GMT which is close to the position of the liquid water maximum observed by the microwave radiometer at the same time. The shape of the cloud mass resembles that of the region of high backscatter at 23.16 GMT.

Some idea of the structure of the upper air in between fronts A and B can be gained from the JASIN radiosonde ascents. On 4 August ascents were made at approximately hourly intervals throughout daylight hours from ships at the corners of the 200-km triangle. Preliminary analysis indicates similar structure at all three corners. The most significant feature is the deep, moist layer in middle levels of the troposphere. At the base of this layer there is a marked temperature inversion such that convection from the surface cannot penetrate above a height of $\sim 2 \text{ km}$. However, the air above would be quite unstable if lifted by a small amount and could support deep convection extending from 2 to 6 km. The inversion marks the frontal surface of a cold occlusion and it is when the surface front passes that the wind veer occurs. Convergence at this feature could release the potential instability aloft.

Upper-air winds were deduced from Loran-C tracking of the sondes and, assuming the convective system to be steered with the mid-tropospheric flow⁷, implied a propagation velocity of $6\text{--}7 \text{ m s}^{-1}$ towards 280° , that is with a significant component parallel to the front. This is consistent with the sequence of cloud photographs and suggests that the feature would be carried over the southern part of the triangle at about 23.00 GMT. The weather logs of the JASIN ships revealed that thunderstorm activity had been reported by three ships in that region between 21.00 GMT and midnight (see Fig. 4 for a synoptic chart).

We conclude that the anomalous SASS winds occurred in a region where deep mid-level convection was taking place. Possible explanations for the unrealistic satellite winds are: (1) that the regions of high backscatter (Fig. 2) are associated with increasing ocean surface roughness due to locally high winds in the vicinity of the convective cells which are small compared with the SASS resolution cell ($18 \times 70 \text{ km}$); (2) the large raindrops in the storm may significantly modify the ocean surface

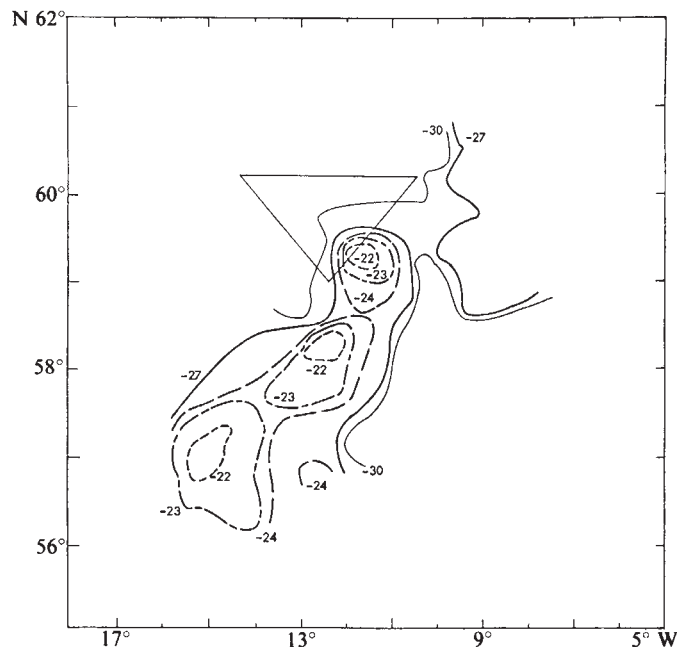


Fig. 2 Contours of normalized backscatter intensity (σ°) obtained from part of the left-hand backward viewing SASS beam in the vertical polarization mode. Rev 557, 23.16 GMT 4 August 1978. Units are Db. Values of σ° have been corrected for variation with incidence angle. To deduce wind direction data from two beams are combined with a consequent loss of areal coverage, hence the difference between Figs 1 and 2.

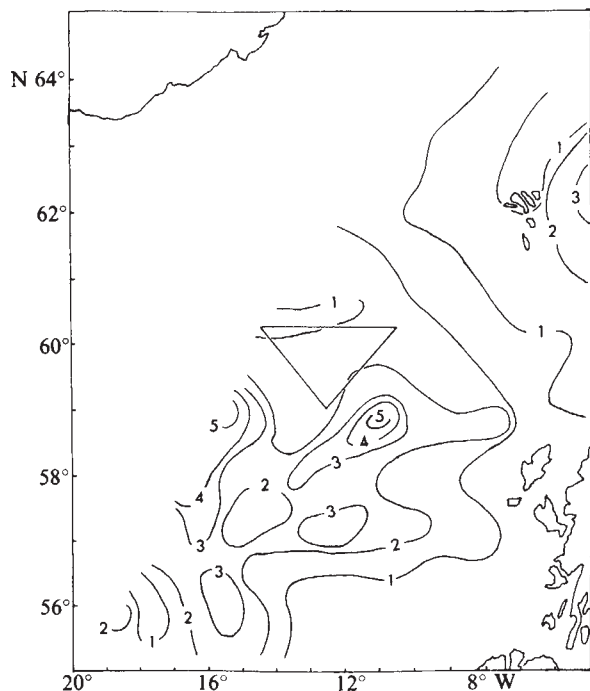


Fig. 3 Contours of vertically-integrated liquid water (units in 0.1 kg m^{-2}) for Rev 556, 21.33 GMT 4 August as deduced from SMMR.

roughness, thereby invalidating the relationship between σ^0 and wind vector; and (3) the rain may be responsible for additional backscatter in the atmosphere.

Concerning explanation (1), although thunderstorms^{8,9} can produce large gusts at the surface through the evaporation of precipitation into the downdraught, on this occasion there was no evidence from surface data sampled at 1-min intervals that gusts exceeded 8 m s^{-1} which is consistent with the observed thermodynamic structure.

For explanation (2), recent radar scattering experiments (Moore, personal communication) conducted in a wind/wave

tank have shown that the backscatter from small-scale surface roughness produced by artificial rain predominates for light wind conditions ($<10 \text{ m s}^{-1}$). To account for the large σ^0 , the affected area would have to be several tens of km^2 with moderate rain rates ($5\text{--}10 \text{ mm h}^{-1}$).

Finally, for explanation (3), calculations show that the large σ^0 could be the result of backscatter from a single, localized rain cell. Assuming a thunderstorm cell size of 80 km^2 and a vertical rain column of 5 km would require rain rates of $\sim 12 \text{ mm h}^{-1}$ to account for the observed σ^0 . Surface observations from ships near the anomaly indicate mainly light rain ($0\text{--}2 \text{ mm h}^{-1}$) but with heavy rain showers at one ship from 23.32–23.45 GMT. It seems that the anomaly was caused by either direct or indirect effects of rain or a combination of the two. Other factors which might lead to high backscatter are hail, which, because of its extremely limited extent is unlikely to have been observed from the ship array, and h.f. interference due to lightning.

Further work is necessary to clarify why SASS fails to give realistic winds. This is of special interest if a combination of different Seasat sensors and ground-based observations indicate that the anomaly is related to an actual atmospheric phenomenon (as above) which otherwise might not be detected. Detailed analysis of the combined JASIN/Seasat data set should improve our understanding of air-sea interaction and the links between the near-surface layers and larger scales.

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Determinations by Seasat of atmospheric water and synoptic fronts

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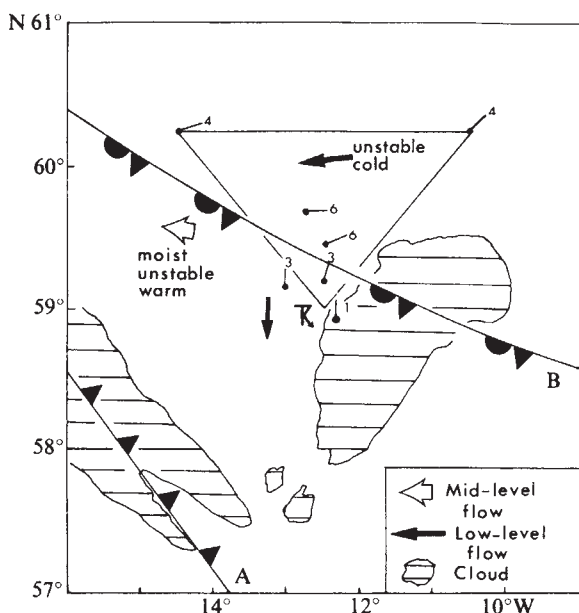


Fig. 4 Synoptic chart showing surface winds in the JASIN area (speeds in m s^{-1}) and the positions of fronts A and B at 22.00 GMT. Areas of deeper cloud, as identified from Seasat VIRR imagery, are also indicated together with information on the characteristics of the flow at mid-levels obtained from radiosonde data in the JASIN area.

The Seasat Scanning Multichannel Microwave Radiometer (SMMR)^{1,2} determined the total atmospheric water vapour, q , over 600-km wide swaths with a resolution of 54 km . Using radiosonde data from the Joint Air-Sea Interaction experiment, JASIN³, we show here that the SMMR q distributions can be used to detect the position of atmospheric fronts in the lower troposphere. Unlike visible and IR radiometry these SMMR determinations are not hampered by extensive cirrus or by lack of frontal cloud. Advantages of the SMMR over previous passive microwave instruments on satellites such as Nimbus 5 and 6 (refs 4–9) are: the use of more channels, allowing better discrimination between the effects of liquid water, water vapour and sea state; and improved spatial resolution. The SMMR performance has been evaluated at several workshops^{10–14}. Our results¹⁵ show that the SMMR q determinations have similar accuracy to *in situ* radiosonde measurements.

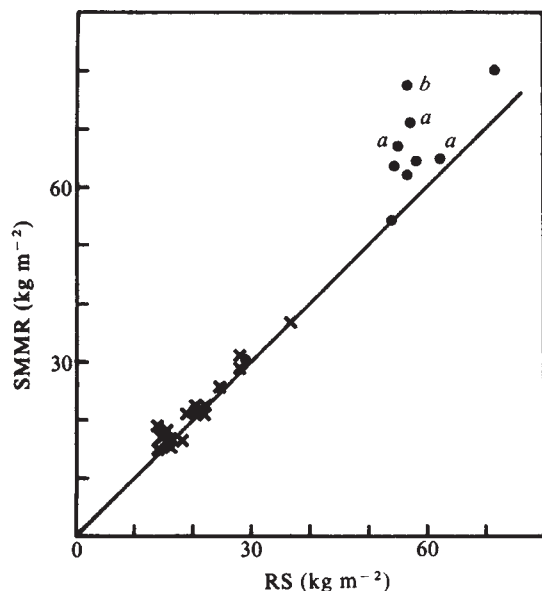


Fig. 1 Comparison of integrated atmospheric water vapour q_t as measured by the SMMR and by radiosondes. $\times$, JASIN values; $\bullet$, tropical soundings. *a* indicates tropical soundings for which SMMR values may be too high due to the island of Guam being within the field of view. *b*, SMMR values may have been affected by heavy rain.

Independently evaluated SMMR and radiosonde estimates of q_t were compared at the Seasat-JASIN Workshop¹³ (Fig. 1). The SMMR results were evaluated using the Wentz algorithm¹⁰, the nearest grid values being linearly interpolated to the radiosonde launch position. Radiosonde data from the JASIN experiment, conducted in the North Atlantic (60°N, 12°W) during July to September 1978³, are shown. Specially calibrated fast sampling radiosondes were used and comparisons restricted to flights launched within ± 2 h of Seasat overpass time. Estimated accuracy of the radiosonde q_t value was $\sim \pm 1.7$ kg m⁻² (ref. 13). The mean difference, SMMR minus radiosonde, was 1.2 ± 1.6 kg m⁻². To provide comparison at higher q_t values

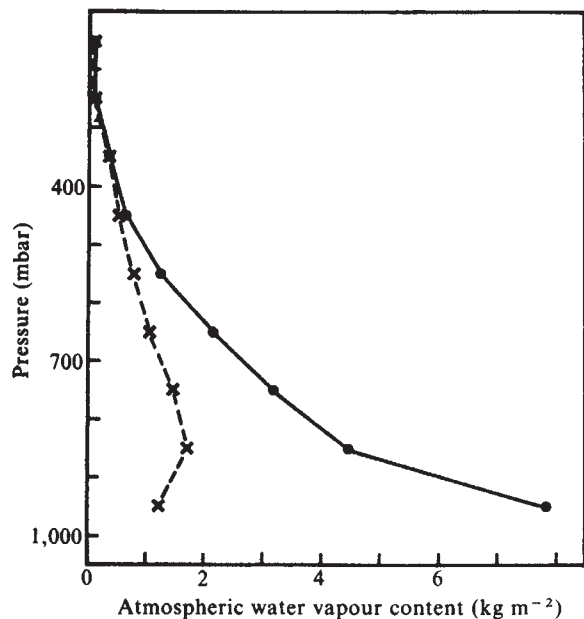


Fig. 2 Mean atmospheric water vapour content of 100-mbar layers during JASIN. Solid line shows the mean value and the dashed line the standard deviation of individual observations for some 200 radiosonde flights of which 110 reached 400 mbar.

radiosonde flights from tropical islands and weather ship Tango are also plotted. These are standard synoptic ascents within 3 h of overpass time. Estimates of q_t cannot be obtained over land and the tropical island of Guam, being significant in size compared with the SMMR resolution, may have affected the data for three flights marked. One comparison may be affected by local heavy rain. Eliminating these flights the mean difference is 4.9 ± 3.7 kg m⁻². Radiosonde errors would be expected to be greater in these ascents and, as for JASIN, the actual error due to the SMMR is not known. However, changes to the SMMR evaluation procedures following the Seasat-JASIN workshop have shown reduced biases¹⁴.

The SMMR also provided liquid water and rain rate estimates; however, no quantitative evaluation was possible because of the difficulty of obtaining reliable *in situ* measurements. The horizontal variation of liquid water content was

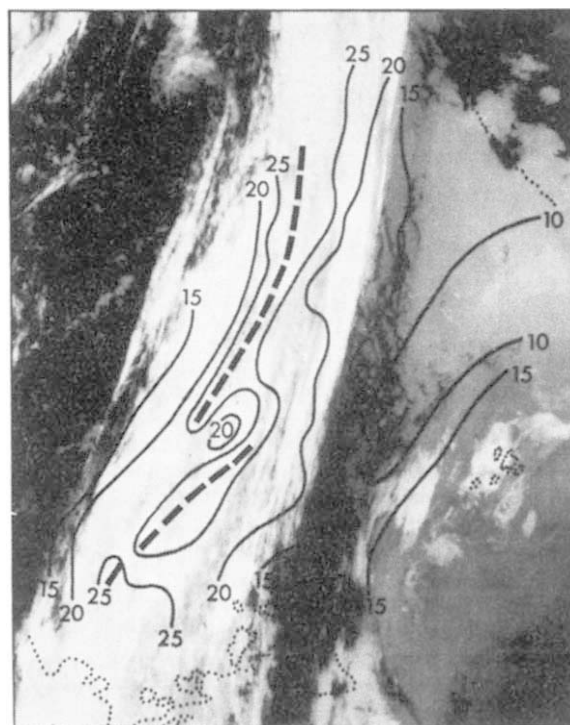


Fig. 3 NOAA 5 IR satellite photograph at 10.37 GMT on 5 September 1978 showing a front stretching from Ireland and Scotland (lower left) to Iceland (upper right). SMMR derived values of q_t are shown in kg m⁻². The dashed line marks the maximum in these values. (Photograph: University of Dundee.)

qualitatively consistent with the meteorological conditions (see ref. 16). For JASIN, SMMR indicated rain when precipitation was 'widespread'—reported by several JASIN ships. Light scattered showers were not detected probably because such showers have horizontal dimensions very much less than the SMMR resolution. Although difficult to obtain¹⁷, quantitative determination of rainfall would have obvious meteorological value. Rain detection is also needed to flag doubtful q_t values (Fig. 1). However, two different rain rate algorithms used in SMMR evaluation gave different quantitative results showing that much more work is needed on this variable.

The accurate horizontal distributions of q_t provided by SMMR are useful not only for the calculation of atmospheric water budgets¹⁸, but also for determining the position of synoptic scale fronts in the lower troposphere. To demonstrate this we need to consider the vertical distribution of water vapour in the atmosphere. Figure 2 shows the mean water vapour content of each 100-mbar atmospheric layer for the whole of the JASIN experiment. The water vapour content falls rapidly with

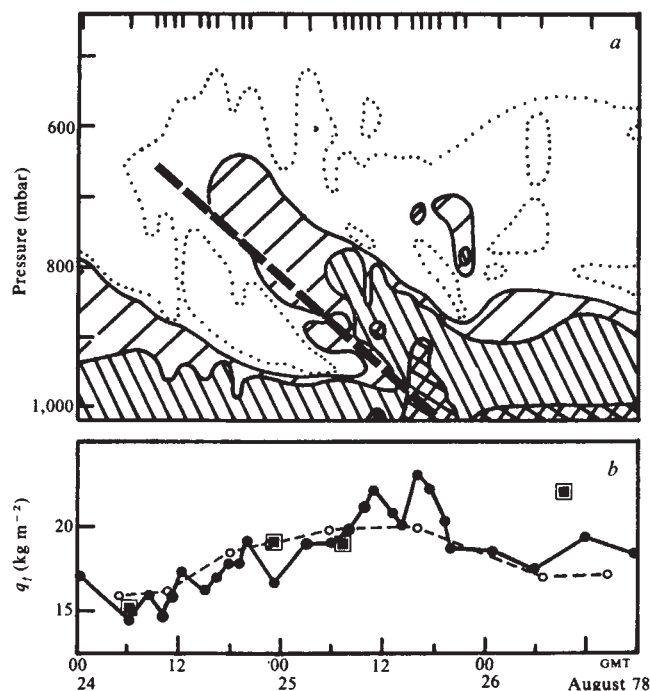


Fig. 4 *a*, Time-height atmospheric cross-section showing the variation of specific humidity with the passage of a warm front. Values shown are 2 g per kg (dotted), 4–6 g per kg (wide diagonals), 6–8 g per kg (close diagonals), above 8 g per kg (cross-hatched). The section is based on 31 radiosonde flights at the times marked at the top. The dashed line marks the approximate region of the frontal surface. *b*, Values of q_t from the radiosonde data (solid line), SMMR (■) and SMMR q_t distribution at 07.30 GMT on 24 August. The moist air ascending the front results in an increase in q_t with time to a maximum at about the time of surface frontal passage. Following the front there is a decrease to the warm air boundary layer value.

decreasing pressure and temperature, ~80% of the total occurring in the lowest 250 mbar. In contrast the variability of water vapour content is a maximum not at the surface but in the 900–800 mbar layer, and decreases less rapidly, 80% of the variation being spread over the lowest 400 mbar. Thus for this mid-latitude area, although the surface boundary layer is typically 1–2 km deep and contains much of the water vapour, the variability of q_t is not dominated by boundary layer processes but reflects changes in humidity at levels up to 5 km. Synoptic scale fronts cause moistening at these levels and hence result in significant variations of q_t . For example, Fig. 3 shows a NOAA 5 satellite IR image in which an occluded front is marked by a 250-km wide band of cirrus which masks any lower level clouds. The variation of q_t measured by SMMR showed a strong maximum associated with the front, marked in Fig. 3 by the broken line. The position of this maximum was well defined to within the 54-km SMMR resolution, except in one area where a more complicated frontal structure was indicated.

The actual position of the surface front relative to the q_t maximum will vary from case to case. As an example Fig. 4*a* shows a radiosonde derived time-height cross-section of specific humidity during the passage of a warm front. Although the front was a significant feature of the radiosonde data, lack of frontal cloud prevented its detection in visible or IR satellite images. The corresponding variation of q_t is shown in Fig. 4*b*. SMMR values are in reasonable agreement with the radiosonde values. A difference on the 26 August was probably caused by larger values of q_t occurring just outside the JASIN area but within the SMMR resolution cell. Radiosonde observations from other JASIN ships show that the front propagated through the area with little change in structure and hence the SMMR horizontal q_t distribution for 07.30 GMT on 24 August has been transformed

to the time variation shown by the dashed line in Fig. 4. Both radiosondes and SMMR show that, in spite of a marked reduction in boundary layer height, there was a gradual increase of q_t with the approach of the front. The surface passage of the front was marked by a sharp decrease of q_t to the warm air boundary layer value. By mapping these q_t variations in the SMMR data from several Seasat orbits the changing position and alignment of the front have been determined more precisely than was possible using the conventional meteorological observations.

Detection of synoptic fronts in the lower troposphere using SMMR q_t measurements offers an important new aid for meteorological analysis which is already proving valuable in the interpretation of the JASIN data.

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Anomalous magnetic directions recorded by laboratory-induced chemical remanent magnetization

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It has been generally accepted that chemical remanent magnetization (CRM) is acquired either in the direction of the ambient field at the time of chemical alteration¹ or in the direction of a pre-existing natural remanent magnetization (NRM)^{2–4}. We report here our experiments in which CRM is carried by magnetite formed during laboratory heating of titanomaghaemite in submarine basalts. This CRM is a stable, single component remanence, acquired in an intermediate direction between the ambient field and pre-existing NRM direction. The role of prior NRM in controlling the direction of CRM is suppressed by externally applied fields of $\approx 50 \mu\text{T}$. (0.5 Oe) but is prominent in fields of $\approx 20 \mu\text{T}$. Thus, stable, intermediate-direction CRMs acquired in past geomagnetic fields are a possibility.

CRM was induced in samples from Site 332B of Leg 37 of the Deep Sea Drilling Project. The chemical alteration that occurs when these basalts are heated above 200 °C has been well documented^{4–7}. The principal magnetic mineral, titanomaghaemite, breaks down forming an intergrowth of a strongly magnetic titanium-poor titanomagnetite, near magnetite in composition and a titanium-rich phase with a composition close to ilmenite. The NRMs of our samples were moderately strong ($> 0.1 \text{ A m}^{-1}$). The Curie temperatures lay between 330 and 365 °C before the laboratory heating. After the samples were heated their Curie temperatures were close to

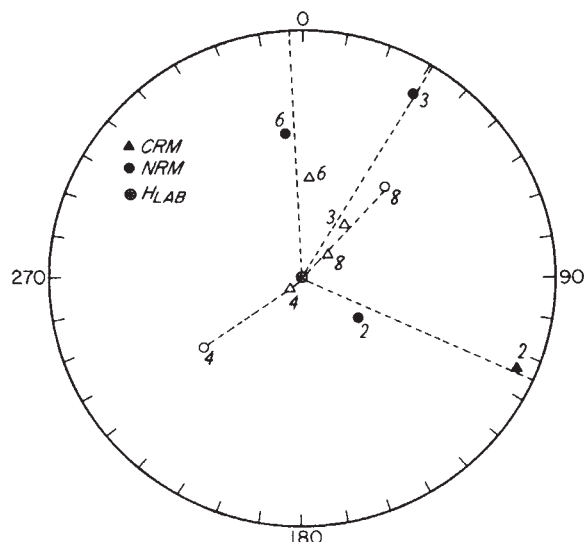


Fig. 1 Equal area plot showing the direction of CRM relative to the laboratory field and the initial NRM direction. Solid (open) symbols denote positive (negative) inclinations. CRM was induced in a 20 μT (0.2 Oe) laboratory field in samples 2 and 4 and in a 10 μT (0.1 Oe) field in samples 3, 6 and 8. The CRM direction is displaced away from the laboratory field direction towards the NRM direction.

580 °C, indistinguishable from the value for pure magnetite. Hysteresis parameters measured before heating indicate that the titanomaghaemite is present as large ($\approx 20 \mu\text{m}$) pseudo-single-domain grains. (The ratio of saturation remanence to saturation magnetization, J_{rs}/J_s , ranges from 0.08 to 0.15.) The magnetite-like phase, which is produced during heating, is present as very fine single-domain grains, having J_{rs}/J_s ratios between 0.33 and 0.36.

CRMs were induced in eight samples. Each 1 cm (o.d.) by

1.5 cm long cylindrical sample was divided into two sub-samples before CRM was induced. The initial NRM was left intact in the first specimen while the second was demagnetized by tumbling in a peak alternating field of 100 mT. The use of two sub-samples allowed us to study separately the roles of the external field and the NRM in determining the direction of CRM. CRM was produced by simultaneously heating both specimens slowly ($\approx 100^\circ\text{C h}^{-1}$) *in vacuo* (10^{-2} torr) to a temperature of 515 °C which was then held for 24 h. Fresh samples were used during successive heatings in laboratory fields of 0, 10, 20 and 50 μT . The fields were applied along the cylindrical axis of each specimen without regard to the NRM direction of the sample. After heating, the samples were cooled in a null field ($< 10^{-2} \mu\text{T}$) to avoid the acquisition of thermoremanent magnetization or TRM.

CRMs acquired in 50 μT all lay along the direction of the laboratory field. The presence of an initial NRM had no apparent effect on the direction of CRM. Strikingly different results were obtained with smaller inducing fields (Fig. 1). In five of the eight samples studied, the CRM in samples with an initially intact NRM did not lie in the laboratory field direction. The CRM directions in these samples were deflected by as much as 110° from the laboratory field direction towards the initial NRM direction. The CRM in samples which were initially demagnetized always lay in the external field direction. All the CRM directions were stable in alternating fields up to 100 mT (Fig. 2). The CRM produced with an intermediate direction is therefore a single, directionally stable component and is not a vector sum of components in the laboratory field and NRM directions. Slight initial directional changes on demagnetization are attributed to the removal of viscous components acquired by fine grained magnetite near superparamagnetic size. The CRMs were magnetically hard with median demagnetizing fields (MDFs) of 57–68 mT (Fig. 3). This contrasts with the magnetically soft NRM of the parent titanomaghaemite in which MDFs ranged from 11 to 15 mT.

Alternating field demagnetization curves of CRM were compared with those of saturation isothermal remanence

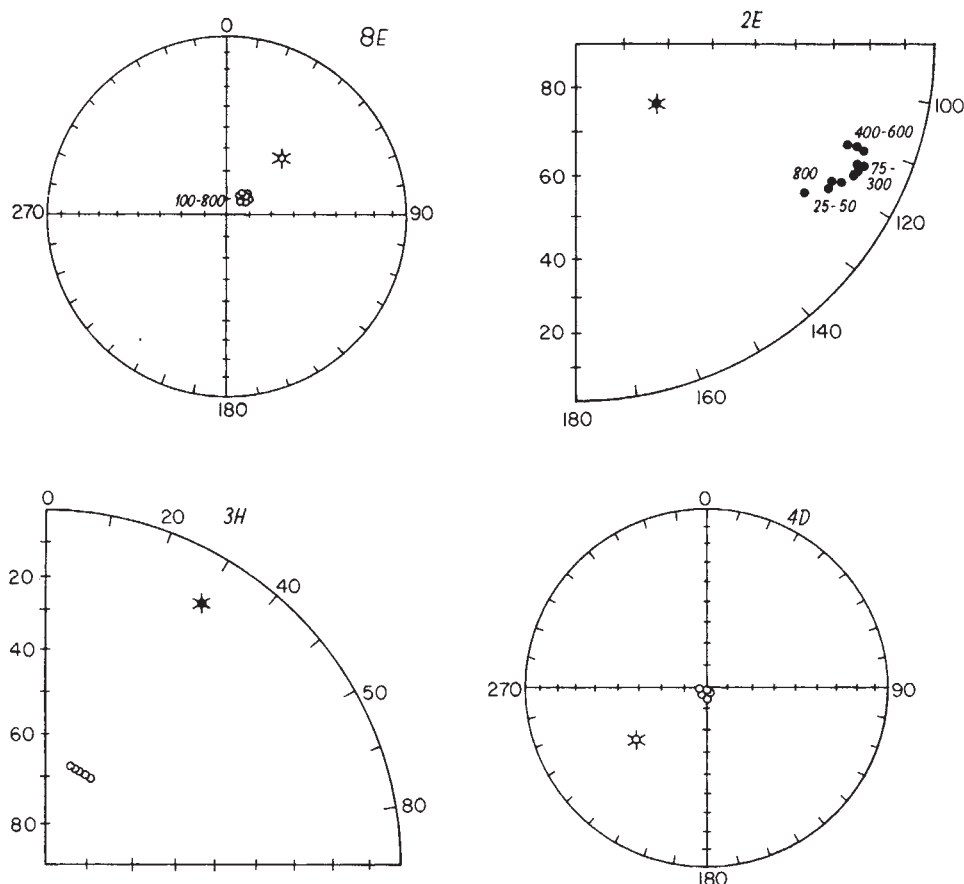


Fig. 2 Directions of CRM during a.f. demagnetization. ☆ Denotes the primary NRM direction. There is no movement towards either the NRM direction or the vertically applied laboratory field direction. This indicates a single component CRM.

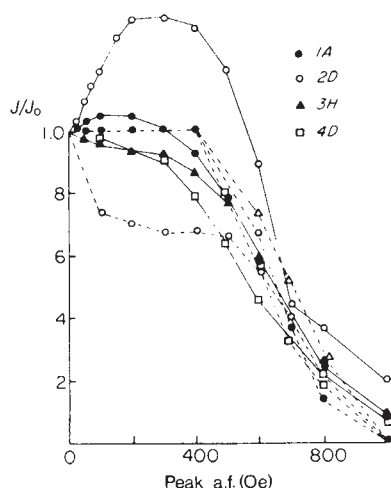


Fig. 3 Intensity of CRM and ARM during a.f. demagnetization. CRM is given by a solid line, ARM by a dashed line.

(SIRM)—and also of anhysteretic remanent magnetization (ARM) acquired in biasing fields of 50 μ T and 100 mT a.f. Both the ARM and the SIRM were partially thermally demagnetized by a rapid heating to 515 $^{\circ}$ C in a null field before a.f. demagnetization was begun. This allowed us to compare the coercivity spectra in the same blocking temperature range for each type of remanence. The a.f. demagnetization curves of CRM, ARM and SIRM are essentially the same at a.f. levels above 40 mT. It would, therefore, be difficult to identify the CRM on the basis of a comparison of coercivity spectra. Differences in the portion of the coercivity spectra below 40 mT result from VRM acquired between the acquisition of CRM and its subsequent measurement. The ARM was demagnetized immediately after acquisition and the samples had little time in which to acquire VRM.

As further chemical alteration accompanied heating to temperatures above 515 $^{\circ}$ C, a direct comparison of the properties of TRM and CRM in the 515–580 $^{\circ}$ C blocking temperature range was not possible.

We conclude that both the ambient magnetic field and pre-existing NRM have a role in determining the direction of CRM. In ambient fields of $< 50 \mu$ T the CRM may lie in a direction between the NRM direction and the ambient field direction. Neither of these directions can be recovered by techniques such as vector subtraction of a.f. demagnetization data.

CRM of this type will be difficult if not impossible to distinguish from other remanences on the basis of a.f. demagnetization curves. A direct comparison of CRM with TRM coercivity spectra was not possible because further chemical alteration accompanies heating above 515 $^{\circ}$ C, but we have found that the a.f. demagnetization characteristics of the CRM are very similar to those of ARM which is often considered an analogue of TRM⁶.

It cannot therefore be assumed that CRM will faithfully record the geomagnetic field direction either at the time of the sample formation or when they alter later. A particularly misleading result could arise when chemical alteration occurs after a geomagnetic polarity reversal. The presence of both NRM and a reversed geomagnetic field could result in a single chemical magnetization component nearly perpendicular to the geomagnetic field direction. This mechanism could be a contributing factor in the anomalously shallow palaeomagnetic inclinations often observed in deep drilled marine basalt samples⁹.

How general are these conclusions? If the direction in which CRM is acquired is partly controlled by magnetostatic interaction with a pre-existing NRM, then the proximity of the parent and daughter magnetic carriers and the intensity of the NRM relative to the external field are critical. If the coupling between the NRM and the CRM is magnetostatic, the moderately high

temperatures used in our experiment will have reduced the influence of the NRM because of the temperature dependence of the spontaneous magnetization of the parent magnetic minerals. In both the low temperature oxidation undergone by submarine basalts and the high temperature alteration of titanomaghaemite which is described here, and which can occur naturally in subaerial basalts¹⁰, the parent and daughter magnetic minerals are closely situated and both are strongly magnetic. Thus, in these cases anomalous CRM directions may be common. If CRM is produced in a system where the parent magnetic mineral is essentially non-magnetic such as when magnetite is grown during the serpentinization of olivine, the NRM of the sample as a whole may be of little importance in controlling the CRM direction.

Our present results do not enable us to decide for or against the magnetostatic model of CRM directional control. Current experiments on CRM acquisition during the reduction of weakly magnetic haematite to magnetite may help us resolve this question.

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Isotopic and biostratigraphical records of calcareous nannofossils in a Pleistocene core

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Knowledge of the species composition is essential for the isotopic record of polyspecific coccolith assemblages in deep-sea sediments to be interpreted properly. We have determined the carbon and oxygen isotopic composition as well as the relative abundance of coccolith species on the 3–25 μ m fractions of the Caribbean core P6304-4. The dominant taxon in all samples is well-preserved *Gephyrocapsa* spp. Oxygen isotope variations clearly define glacial-interglacial oscillations. Except in the upper part of the core, carbon isotopic compositions are not related to the oxygen isotope stratigraphy. The occurrence of three coccolith datum levels in the core correlates almost exactly with globally-synchronous horizons¹. The amplitude of the coccolith $\delta^{18}\text{O}$ record (2.4‰) is significantly greater than that for the planktonic foraminifera *Globigerinoides sacculifer* in P6304-4 and adjacent cores^{2–4}. We suggest here that selective dissolution or deep calcification during interglacials reduced the amplitudes of *G. sacculifer* in these cores and that the coccolith record is a more reliable indicator of temperature and $\delta^{18}\text{O}$ changes in surface seawater.

Calcareous nannofossils (dominantly coccoliths) comprise about half of the CaCO_3 in deep-sea sediments⁵. Because of their abundance, shallow and restricted depth habitat⁶, wide geographical distribution⁶, and dissolution-resistant characteristics^{7–9}, the stable isotope record of calcareous nannofossils

should complement other techniques in palaeoceanographic investigations.

The oxygen and carbon isotopic composition in coccolith-rich fractions from sediments ranging in age from Upper Cretaceous to Recent have been used as indicators of sea-surface conditions¹⁰⁻¹⁷. Results suggest that coccolith fractions yield isotopic records which are generally consistent with data on foraminifera for the same time interval (and often on the same core material). Nonetheless, a major problem remains in interpreting coccolith isotopic compositions: because of their very small size, coccoliths can only be concentrated from deep-sea sediments as polyspecific assemblages. Because species-dependent non-equilibrium isotopic fractionation in coccolith secretion has been demonstrated^{18,19}, it is possible that variations in species abundance will obscure the palaeoenvironmental significance of the isotopic signal from coccolith assemblages. To evaluate this possibility and to test the usefulness of coccolith isotope stratigraphy in Pleistocene sediments, we have determined the isotopic composition and coccolith taxonomy in the fine-fraction of core P6304-4 from the Caribbean.

Core P6304-4 is one of four long piston cores from the same area of the central Caribbean (~15° N, ~70° W, 3,930-4,140 m in depth) on which Emiliani^{2,3} has reported the oxygen isotope record of *G. sacculifer*. We separated the 3-25 µm fraction of bulk sediment samples taken at 10-cm intervals by settling and short centrifugation techniques²⁰. High-resolution light microscopy and scanning electron microscopy were used to estimate

the relative abundance and state of preservation of 34 coccolith species. Details of our taxonomic data will be given elsewhere (T.F.A. and J.C.S., in preparation). These results indicate that the carbonate fraction in the 3-25 µm fractions consists almost exclusively of well-preserved coccoliths. The most abundant taxon at all depths was the genus *Gephyrocapsa*; *G. caribbeanica* and *G. oceanica* were dominant (Fig. 1). *Emiliana huxleyi*, an abundant species during the past 270,000 yr (refs 1, 21), was absent or rare in our 3-25 µm fractions (but was abundant in the bulk sediment samples). Because of its relatively small size, *E. huxleyi* was not retained in the 3-25 µm fraction. Total (coccolith) carbonate makes up only 10-50% of this fraction. X-ray diffraction analysis indicates that the non-carbonate portion consists of quartz, chlorite and/or kaolinite, mica (illite?) with occasional feldspar and smectite.

The samples were treated with 100% H₃PO₄ at 25 °C to evolve CO₂ for mass-spectrometric isotope analysis. Pretreatment consisted of soaking the bulk sediment in a 5% clorox solution before size fractionation. The samples were not vacuum roasted as initial experiments indicated that this did not improve the precision of replicate analyses (± 0.04 for $\delta^{13}\text{C}$, ± 0.06 for $\delta^{18}\text{O}$) nor alter significantly the isotopic composition of these coccolith fractions. The mass-spectrometric data are reported in δ -terminology as ‰ deviations from the PDB standard²².

The oxygen isotope results show the characteristic 'saw-toothed' pattern of glacial-interglacial oscillations down to at least isotope stage 14 (Fig. 1). The sharp glacial-to-interglacial

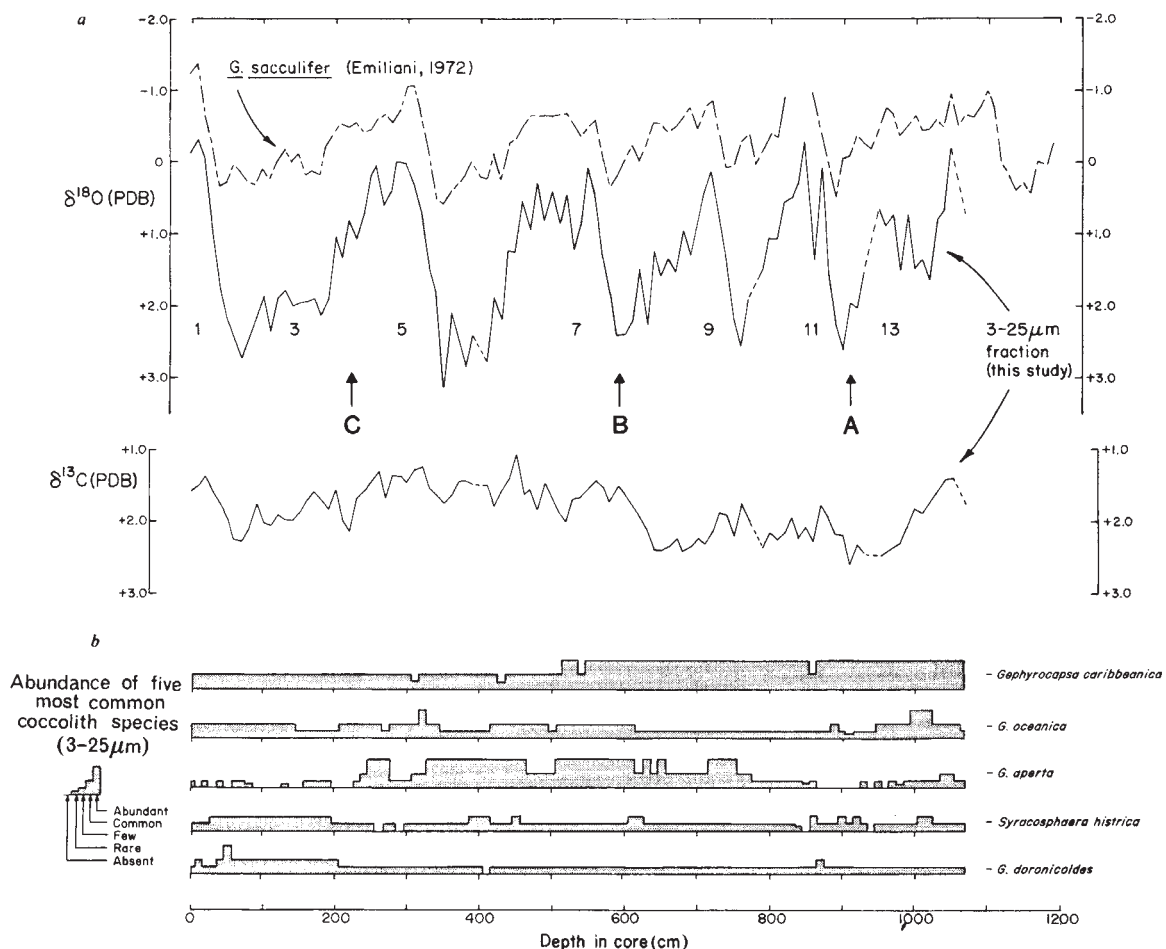


Fig. 1 The oxygen and carbon isotopic results on the 3-25 µm fraction of core P6304-4. Emiliani's² oxygen isotope data on *G. sacculifer* is also shown. The numbers beneath the 3-25 µm $\delta^{18}\text{O}$ curve identify the interglacial isotopic stages³. In *a*, A, B and C indicate the globally-synchronous late Quaternary coccolith datum levels of Thierstein *et al.*¹. A, extinction of *Pseudoemiliana lacunosa* (458,000 yr BP) at 910 cm; B, first appearance of *E. huxleyi* (268,000 yr BP) at 590 cm; C, reversal in dominance of *G. oceanica* and *E. huxleyi* (85,000 yr BP in tropical and subtropical cores) at 220 cm. The relative frequency of the five most abundant coccolith species in the 3-25 µm fraction (*b*) was determined from the examination of about 100 fields of view on smear slides⁴⁵. Abundant, 10-100 specimens per field of view; common, 1-10 specimens per field of view; few, single specimen in 1-10 fields of view; rare, single specimen in 10-100 fields of view.

Table 1 $\delta^{18}\text{O}$ maxima and minima (stages 1–12) for *G. sacculifer* from Caribbean cores^{2–4,35}

Isotope stage	A172-6 4,160 m 14°59' N 68°51' W	P6304-4 4,136 m 15°27' N 70°43' W	P6304-9 4,126 m 14°57' N 68°55' W	P6304-7 3,929 m 15°06' N 69°48' W	P6304-8 3,927 m 14°59' N 69°20' W	A179-4 2,965 m 16°36' N 74°48' W	V12-122 2,800 m 17°00' N 74°24' W
1	-1.87	-1.30	-1.36	-1.38	-1.29	-1.79	-2.18
2	-0.05	+0.30	+0.43	+0.29	+0.59	+0.12	0.00
5	-1.39	-1.05	-1.36	-1.32	-1.34	-1.65	-1.69
6	-0.10	+0.57	+0.39	+0.30	+0.43	+0.11	+0.24
7	-1.41	-0.67	-1.22	-1.15	-1.15	-1.63	-1.53
8	-0.23	+0.25	+0.39	+0.11	+0.34	+0.25	-0.03
9	-1.42	-0.81	-1.23	-1.49	-0.89	-1.60	-1.86
10	+0.03	+0.07	+0.22	+0.21	+0.32	-0.13	-0.06
11	-1.08	-0.95	-0.82	-1.14	-0.72	-1.40	-2.14
12	(-0.62)	+0.49	+0.21	+0.41	+0.47	(-0.71)	+0.33
δ_{igl}	-1.4	-1.0	-1.2	-1.3	-1.1	-1.6	-1.9
δ_{gl}	-0.1	+0.3	+0.3	+0.2	+0.4	0.0	0.0
$\Delta\delta$	1.3	1.2	1.5	1.5	1.5	1.6	1.9

δ_{igl} , Mean interglacial. δ_{gl} , Mean glacial. $\Delta\delta$, Average glacial–interglacial amplitude for adjacent stages.

transitions are well-defined. These stage boundaries in the coccolith fraction occur 10 (± 15) cm lower than in Emiliani's *G. sacculifer* record². Given that this difference is about the same as our sampling resolution, the agreement is excellent. In addition, the occurrence of three coccolith biostratigraphic markers relative to the coccolith $\delta^{18}\text{O}$ curve is in almost exact agreement with their correlation in nine other deep-sea sediment cores to the late Quaternary oxygen isotope chronostratigraphic framework^{1,23}. The agreement is slightly better for the coccolith record than for the foraminifera record. This correlation between biostratigraphic and isotopic results is convincing evidence for the reliability and precision of the stratigraphical information in the $\delta^{18}\text{O}$ record of coccoliths.

Variations in the carbon isotopic composition of the coccolith fraction (+1.1 to 2.6‰) are not obviously related to the oxygen isotope signal. A possible exception is in the interval of the most recent deglaciation, where $\delta^{13}\text{C}$ values decrease by 0.8‰ from the Upper Pleistocene to the Holocene. A similar carbon isotope shift is recorded in planktonic foraminifera from the Caribbean core V12-122²⁴. These results suggest upwelling of nutrient- and ^{12}C -rich waters was higher in the Caribbean during the Holocene than during the last glacial maximum; this is opposite to changes in vertical circulation inferred from carbon isotope evidence in cores from the eastern subtropical Atlantic²⁵ and the western equatorial Pacific¹⁴.

Notwithstanding the obvious similarity in isotopic stratigraphy, there are two prominent differences between the $\delta^{18}\text{O}$ signals of the coccolith fraction and *G. sacculifer* in core P6304-4. First, coccoliths are enriched in ^{18}O relative to *G. sacculifer* by 1 to over 2‰. Part of this discrepancy could be due to differences in standard calibrations and sample preparation techniques between laboratories. On the other hand, this difference is consistent with data on oxygen isotopic fractionation in these taxa. *G. oceanica* grown in culture experiments is enriched in ^{18}O with respect to equilibrium¹⁹. The question of whether *G. sacculifer* deposits its test at oxygen isotope equilibrium with near-surface seawater has not been resolved. *G. sacculifer* assemblages from well-preserved Holocene sediments are often close to isotopic equilibrium^{2–4,14,26,27}. In contrast, living *G. sacculifer* populations collected in plankton tows are depleted in ^{18}O relative to isotopic equilibrium with ambient seawater^{24,28–31}. In either case, provided that the isotopic properties of *G. oceanica* are representative of the coccolith fraction in P6304-4, then the direction of the difference in $\delta^{18}\text{O}$ between this fraction and *G. sacculifer* is reconciled. (Comparable differences between coccoliths and planktonic foraminifera have been reported previously^{13–16}.)

Second, the amplitude of glacial–interglacial fluctuations averages 2.4‰ for the coccolith fraction through stage 12 but only 1.2‰ for *G. sacculifer*. Inasmuch as this shallow-dwelling foraminifera occupies the same approximate depth habitat as coccolithophores^{26,27,32}, both carbonate constituents should

have been exposed to the same temperature and oxygen isotope composition of seawater during climatic cycles and therefore should have recorded the same $\delta^{18}\text{O}$ fluctuations. Obviously the isotopic record of one (or perhaps both) of these carbonates has been affected systematically by one or more other factors such as variations in non-equilibrium isotope fractionation, changes in depth habitat, and isotopic bias due to selective dissolution³³.

It is possible that changes in the abundance of the most common species contributed to the glacial–interglacial $\delta^{18}\text{O}$ fluctuations of the coccolith fraction. Our taxonomic study proves that they did not (Fig. 1). *Gephyrocapsa* is the dominant taxon in the 3–25 μm fraction throughout the core. There is no obvious correlation between the abundance of the most common species of this genus, *G. caribbeanica*, *G. oceanica*, and *G. aperta* (and the next most abundant taxa) and the $\delta^{18}\text{O}$ signal of this fraction. Variation in depth habitat of coccolithophores between glacial and interglacial times is implausible. For this effect to operate, coccolithophores would have had to migrate to deeper waters during glacials. However, as they are photosynthesizers their depth habitat is restricted to the photic zone regardless of climate-induced changes in the physical properties of surface seawater. For the same reason, selective dissolution of coccoliths (for which there is no evidence in our samples) would not result in a bias towards an ^{18}O -enriched sediment assemblage as it often does in shallow-dwelling planktonic foraminifera^{27,32–34}. The effect of differences in seasonal temperature contrast between glacial and interglacial times on the $\delta^{18}\text{O}$ record of coccoliths is difficult to evaluate but was probably not of major importance. Temperature estimates based on quantitative faunal analyses^{35–37} suggest that seasonal contrasts during glacials (4–5 °C) was greater than during interglacials (1–2 °C) due to greater variations in winter temperatures. The corresponding maximum seasonality effect on the $\delta^{18}\text{O}$ range of coccoliths (secretion during glacial winters and interglacial summers) would be $\sim +0.4\%$. The actual seasonality influence was probably much smaller. These considerations suggest that the oxygen isotope variation in the coccolith fraction from P6304-4 records primarily climate-induced fluctuations in the temperature and isotopic composition of Caribbean surface seawater.

If this is correct, then the amplitude of the $\delta^{18}\text{O}$ signal in *G. sacculifer* from P6304-4 has been truncated. The isotopic record of *G. sacculifer* in other Caribbean cores analysed by the University of Miami^{2–4} are generally consistent with the results on P6304-4 (Table 1, columns 1–6). However, the amplitudes of the $\delta^{18}\text{O}$ signal in the five deeper cores (>3,900 m), including P6304-4, are smaller than the amplitudes recorded in two shallower cores (<3,000 m). This difference in amplitude is due mostly to less negative interglacial $\delta^{18}\text{O}$ values in the deeper cores versus the shallower cores. The amplitude of the coccolith isotope signal in P6304-4 (2.4‰) is more compatible with the results from V12-122. In fact, the $\delta^{18}\text{O}$ signal predicted from

the isotopic and faunal temperature data on this core³⁵ together with experimental fractionation results on *Gephyrocapsa*¹⁹ is in very good agreement with our analyses (unpublished data).

The differences in the oxygen isotope record of *G. sacculifer* among these Caribbean cores, first noted by Shackleton *et al.*²⁸, is not easily explained. The relationship between $\delta^{18}\text{O}$ amplitude and depth would suggest that selective dissolution of *G. sacculifer* during interglacial epochs resulted in a variable proportion of ^{18}O -enriched tests in the carbonate fraction from which the samples were picked. Shackleton and Opdyke^{23,38} report a similar relation between the $\delta^{18}\text{O}$ record of *G. sacculifer* and depth in two cores from the western equatorial Pacific. However, differential dissolution causing a significant increase in the $\delta^{18}\text{O}$ of *G. sacculifer* should also have been obvious in the state of preservation of the coccoliths (J. S. Killingley, personal communication). In addition, poor preservation of foramineral assemblages and low carbonate contents in Quaternary Caribbean and equatorial Atlantic cores are more pronounced during glacial than interglacial stages³⁹⁻⁴². An alternative explanation may involve glacial-interglacial differences in the range of depth habitats and hence the mean temperature of test secretion^{31,42-44}. For example, Duplessy *et al.*³¹ have reported recently that late-stage calcification of *G. sacculifer* during descent into cold waters significantly enriches fossil tests in ^{18}O relative to their living counterparts and note that this effect can produce sedimentary assemblages coincidentally close to isotopic equilibrium with near-surface seawater. We emphasize that oxygen isotopic variations in Pleistocene coccoliths not only provide an excellent stratigraphic record but also may be a more reliable indicator of the temperature and $\delta^{18}\text{O}$ of surface seawater than planktonic foraminifera. In fact coccolith $\delta^{18}\text{O}$ variations may prove to be important for evaluating the extent and effects of the dissolution and/or deep-water calcification of planktonic foraminifera.

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Extensive regulatory capabilities of a *Drosophila* imaginal disk blastema

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Every organism develops body patterns that are accurately repeated among individuals of the same species. To understand how cells interact to form these patterns, many investigators have studied the re-establishment of patterns following genetic and/or physical perturbations. In regulating systems, missing structures may be replaced by cell proliferation (epimorphosis) or by respecification without growth (morphallaxis)¹. Epimorphosis involves the formation and growth of a blastema², defined for vertebrate regeneration as a zone of proliferating cells, localized near the wound surface³, which becomes capable of regenerating even when isolated from the rest of the limb⁴. Clonal analysis of *Drosophila* imaginal disk fragments indicates that during pattern regulation most dividing cells are located near the wound surface⁵. Furthermore, mitotic figures (L. C. Abbott, personal communication) and cells incorporating ³H-thymidine⁶ are clustered near the wound surface of regulating fragments. Here we describe experiments involving cultures of this zone of proliferating cells, and report that it can regulate in isolation. These observations provide evidence that regulation of disk fragments is accomplished by growth of a blastema. Surprisingly, the isolated blastema regenerated more pattern elements than predicted by the polar coordinate model⁷. We resolve this paradox by proposing that regulation is initiated at free cut edges, where a blastema is formed that adds new positional values in a sequence. On completion of wound healing, blastema growth continues until remaining positional disparities are eliminated by intercalation, as presented in the polar coordinate model.

We used the property that imaginal disk tissue differentiates when transplanted into a metamorphosing larva to determine the fate of particular first leg disk regions (Fig. 1a, b). The three-quarter lateral plus endknob fragment (3/4L+EK) differentiated sectors A, B, C and D plus the tarsus. This region of the disk was subdivided into the 1/8LM and 5/8L+EK fragments, which produced sector D and sectors A, B and C plus the tarsus, respectively. Figure 1c gives the sectorized fate map of the disk obtained by pooling results from different fragments. The growth period of disk tissue is extended by transplantation into the abdomen of an adult female (culture *in vivo*). When 3/4L+EK fragments were cultured in adults and then differentiated in larval hosts, they usually duplicated the structures found in the fate map of the fragment (Fig. 1d). The 1/8LM fragment did not regenerate after 6 days in adult culture. An unusually high number of implants were not found (38/62), and the 11 differentiated implants only contained structures seen in the fate map of the fragment.

When the 3/4L fragment duplicates, the prospective sector D contains a zone of proliferating cells⁵. We isolated this area fragment I from 3/4L+EK fragments after 1 day of adult culture (Fig. 2a). The position of the nerve stalk and the

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Fig. 1 *a*, Structures differentiated by the first leg disk. To visualize all the adult structures the leg is displayed as if split open longitudinally. Disks differentiated all the segments and structures found in an *in situ* prothoracic leg. Donors and hosts (La) were late third-instar larvae. We divided the leg cuticle into sectors A–F to facilitate discussion of the results (see ref. 14 for a complete description of the markers). Each sector contained a minimum of two sector-specific markers. Pt = prothorax, Co = coxa, Tr = trochanter, Fe = femur, Ti = tibia, Ta 1 = first tarsal segment, Ta 2–5 = tarsal segments 2–5. * Denotes position of the nerve stalk. Solid arrowheads symbolize metamorphosis. *b*, Fate mapping of disk regions. L = lateral, LM = lower medial, EK = endknob. The height of each column indicates the percentage of the cases that differentiated the particular sector. *c*, Sectorized fate map of first leg disk. *d*, Adult culture of 3/4L + EK fragments. Fragments were injected into the abdomens of fertilized, recently eclosed females (Ad); after 4 days of culture *in vivo* implants were transplanted into larval hosts. Reversed letters indicate duplicate sectors. Forty per cent of the 3/4L + EK fragments only duplicated structures expected from the fate map, 53% also regenerated sector E and 7% only regenerated sector E, without duplicating any structures.

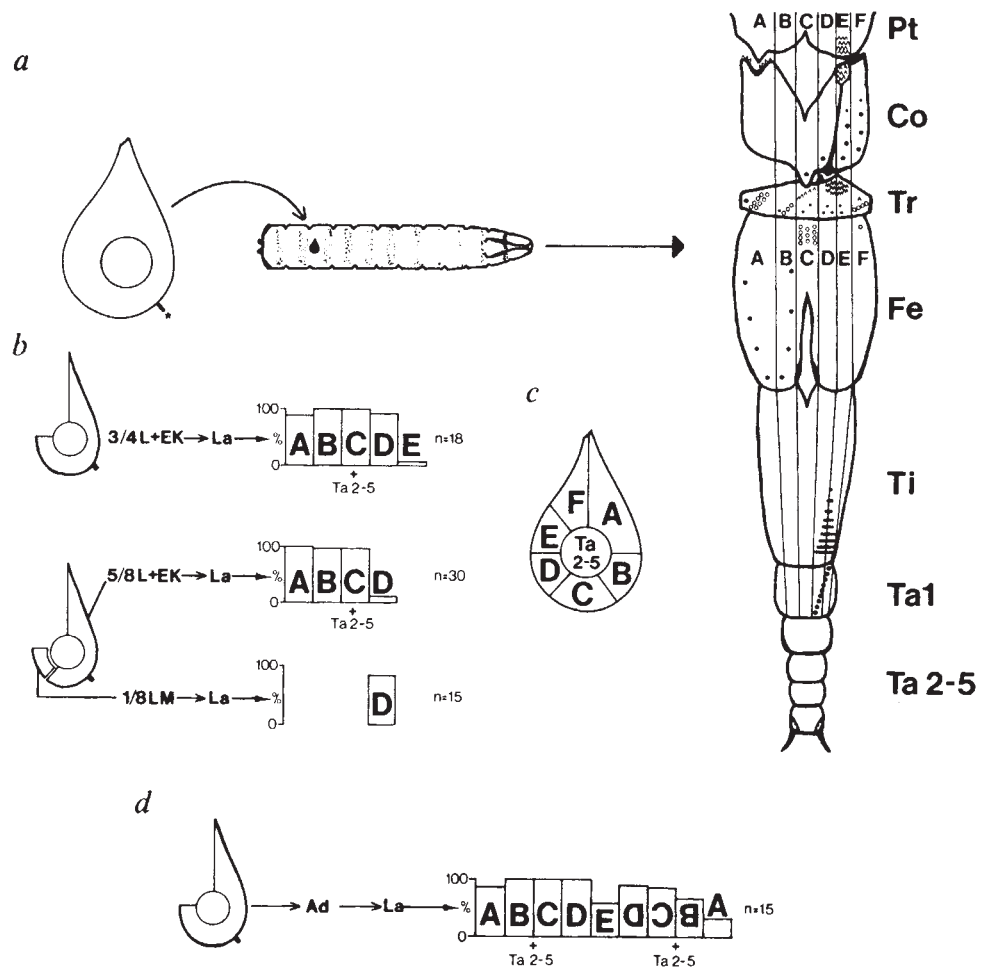
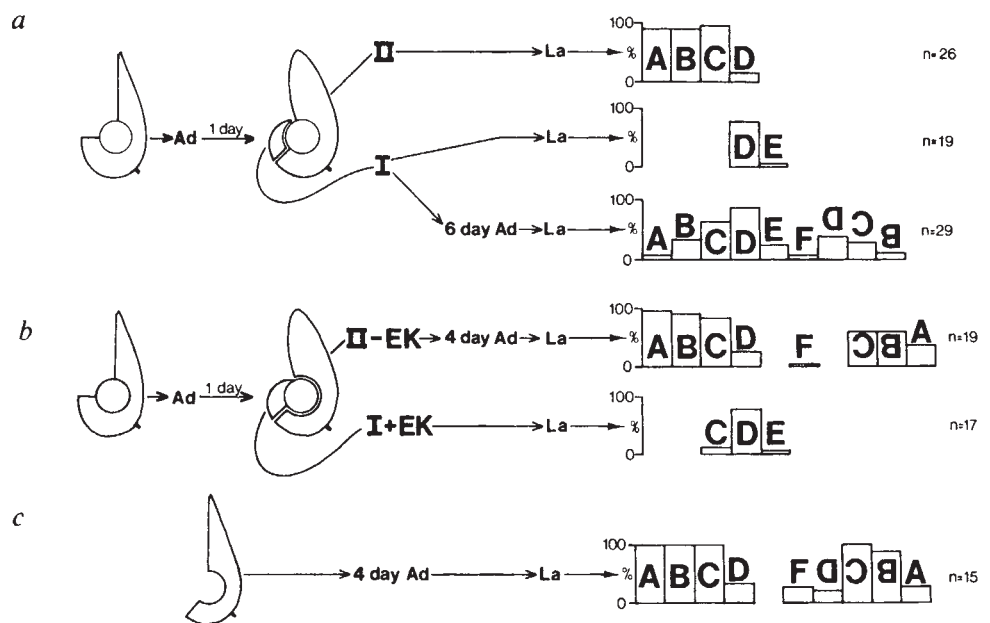


Fig. 2 *a*, Regulatory potential of the blastema region. 3/4L + EK fragments were cultured *in vivo* for 1 day; the implants were then cut into fragments I and II. Immediate metamorphosis was used to determine the fate of the II fragments. Only 15% of these cases differentiated sector D, whereas over 90% produced sectors A, B and C and Ta 2–5. I fragments were either injected into larval hosts or given an additional 6 days of adult culture before metamorphosis. Ta 2–5 appeared only after culture *in vivo* (in 21% of the cases). *b*, Regulatory potential of the II–EK fragment. 3/4L + EK fragments were cultured *in vivo* for 1 day, then I + EK fragments were injected into larval hosts while II–EK fragments were given another 4 days of adult culture before metamorphosis. Ta 2–5 were differentiated by 94% of the I + EK fragments and 37% of the II–EK fragments. Forty-two per cent of the II–EK fragments only duplicated sectors A, B and/or C, 32% also regenerated sectors D and/or F, and 5% only regenerated sector D and/or F. II–EK fragments were used (instead of II fragments) to allow a direct comparison with the I fragment, which also did not include the endknob. Four days of culture were chosen to reduce the high frequency of transdetermination found in this fragment after 6 days of culture. *c*, Adult culture of 5/8L fragments. Fragments were given 4 days of adult culture before metamorphosis. Fifty-four per cent only duplicated sectors A, B and/or C, and 46% also regenerated sectors D and/or F. Ta 2–5 were differentiated by 60% of the cases. See Fig. 1 legend for a description of symbols.



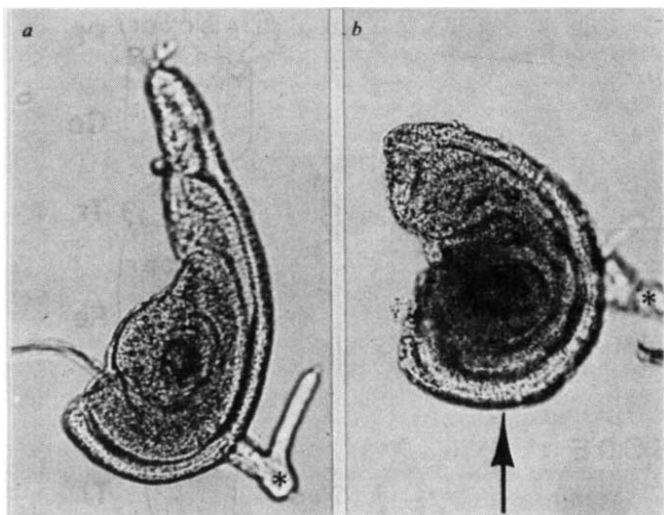


Fig. 3 3/4L + EK fragments immediately after fragmentation (a) and after 1 day *in vivo* culture (b). $\times 178$. * Denotes nerve stalk, arrow indicates position of second cut (see Fig. 2b). Note in b that the original cut surfaces have not fused.

presence of unfused cut surfaces facilitated orientation of the implants (Fig. 3). Immediate metamorphosis of fragment I yielded structures of sector D, but never sector C. The remaining piece (fragment II) predominantly differentiated sectors A, B and C plus the tarsus (Fig. 2a).

We determined the regulatory capabilities of regions within the 3/4L + EK fragment after 1 day of culture *in vivo*. I fragments were isolated, then given an additional 6 days in adult hosts (Fig. 2a). Although the amount of regulation varied, these fragments differentiated identifiable and organized patterns. Most (18/29 cases, 62%) contained structures that were never formed by uncultured I fragments (sectors A, B, C and/or F). Thirteen of these cases duplicated some or all of the structures present (for examples, see Fig. 4, cases 4 and 5). We also analysed these data in a pairwise manner to determine whether these surprising results were caused by cutting errors. Figure 4 shows cases in which both the I and II fragments from a single 3/4L + EK implant differentiated. In cases 1–3, fragment I produced sector D and showed some regeneration. In all the other paired cases I fragments extensively regenerated missing structures. They contained structures also found in the II fragment of the pair (boxed sectors), and in addition were able to differentiate structures that were not part of the 3/4L + EK fate map (sector F, cases 6–8). In case 8 the I fragment produced an entire leg! Thus, we conclude that the I fragments regenerated during culture *in vivo*.

II-EK and 5/8L fragments differentiated the same structures after transplantation into larvae (sectors A, B and C, data not presented) and therefore have the same fate. After 4 days in adults they regulated similarly; most cases duplicated and some of these fragments also regenerated (Fig. 2b, c). In contrast, the I and 1/8LM fragments had the same fate (sector D, Figs. 2a, 1b), but only the I fragment regenerated after 6 days of adult culture. Therefore, during 1 day of culture *in vivo* of the 3/4L + EK fragment the regulative potential of only prospective sector D cells was altered. Furthermore, clonal analysis indicated that cell divisions increased significantly during regulation only in sector D of the 3/4L fragment⁵. We conclude that during 1 day of adult culture a blastema is formed in this region, and we propose that blastema formation and growth are integral steps in the regulation of disk fragments.

The extensive regulatory capabilities of the blastema region challenge current explanations of how imaginal disk fragments regulate. According to the polar coordinate model⁷, wound healing juxtaposes cells with disparate positional values; this stimulates growth, and intercalation proceeds until positional values are continuous. The 'rule of intercalation by the shortest

route' predicts the type of regulation a particular fragment will express. A fragment containing more than half the total number of positional values regenerates, whereas a fragment with less than half the values will only duplicate. The isolated blastema fragments violate two aspects of this rule. First, many I fragments were able both to regenerate and duplicate, which is inconsistent with the model^{8,9}. Second, the I fragments consistently regenerated missing structures, and produced an entire leg in two of the cases given 6 days additional culture *in vivo*, although they contained few positional values. When the 3/4L + EK fragment was cultured *in vivo* for 1 day, on immediate metamorphosis no I fragment duplicated structures, and only 5% differentiated sector E in addition to D (Fig. 2a). According to the positional value map of the first leg disk¹⁰, sectors D and E contain much less than half the number of circumferential positional values.

A possible resolution of this problem is suggested by comparing the time course of wound healing with our data. An imaginal disk consists of a folded sheet of columnar epithelial cells and an overlying sheet of squamous epithelial cells (peripodial membrane), which form a continuous epithelium¹¹. Reinhardt and Bryant¹² have shown that within 12 h of fragmenting a wing disk, the columnar epithelial cells at each cut surface make contact with cells of the peripodial membrane (heterotypic healing). By 1–2 days after fragmentation, columnar epithelial cells from the previously separated cut edges fuse (homotypic healing). The polar coordinate model^{7,12} suggests that homotypic contacts initiate and control pattern regulation. However, we isolated blastemas with extensive regulative capacities before wound healing was complete, and therefore conclude that homotypic closure is not necessary for the initiation of regulation.

Two independent observations support this conclusion and further characterize the early blastema. First, mitotic figures are localized to the blastema region of the 3/4L + EK fragment within 1 day of adult culture (L. C. Abbott, personal communication). Thus, proliferation is stimulated before wound healing is complete. Second, in many regulating leg disk fragments new structures appear in a sequence that originates from the cut surface containing the blastema^{5,13}. This same sequence

		A	B	C	D	E	F
1	I	-	-	-	+	+	-
	II	+	+	+	-	-	-
2	I	-	-	±	+	-	-
	II	+	+	+	-	-	-
3	I	-	-	±	+	+	-
	II	+	+	+	-	-	-
4	I	-	+	+	+	+	-
	II	+	+	+	-	-	-
5	I	-	+	+	+	+	-
	II	+	+	+	+	-	-
6	I	-	+	+	+	+	(+)
	II	+	+	+	-	-	-
7	I	+	+	-	-	+	+
	II	+	+	+	+	-	-
8	I	+	+	+	+	+	+
	II	+	+	+	-	-	-

Fig. 4 Pairwise analysis of the regulatory potential of the presumed blastema area. 3/4L + EK fragments were cultured for 1 day *in vivo*; the implants were then cut into fragments I and II. I fragments were given an additional 6 days in adults before metamorphosis, whereas II fragments were injected into metamorphosing hosts. I and II fragments from the same 3/4L + EK implant were analysed in a pairwise manner. +, Sector present; -, sector absent; ++, sector present and duplicated. Sector considered present when two or more markers were identified. (+), One marker identified. Boxes indicate that the same structures were differentiated by both fragments. Underlined sectors indicate those clearly regenerated.

is followed by isolated blastemas. Figure 2a shows that I fragments cultured in adults for 6 days regenerated and/or duplicated sector B less frequently than sectors C and D. This sequence also is expressed in each case that regulates. For example, when sector B was regenerated, sector C was always present. Therefore, the information necessary for regulating, in a sequence, is present before the completion of wound healing.

Later in the regulative process, homotypic healing is completed and cells at the cut edge are confronted with new neighbours. We propose that blastema growth continues, and additional positional values are intercalated according to the shortest route, as expressed in the polar coordinate model. This proceeds until continuity is restored to the positional field.

Our study indicates that stimuli other than homotypic wound closure, such as heterotypic contacts or simply the presence of a free cut edge, could initiate regulation. We do not know what types of interactions initially determine whether a blastema will regenerate or duplicate. However, we hope our study stimulates new thoughts about how cells interact during pattern formation.

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Estimates of dinosaur speeds from a new trackway site in Texas

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A method for estimating the speed of a dinosaurian trail-maker from the size of its footprints and stride has been described by Alexander¹. However, when applied to known trackways, this method gave rather low speeds (1.0–3.6 m s⁻¹). Russell and Béland² estimated a speed of 1.77 m s⁻¹ for a slowly moving ornithomimid and 7.54 m s⁻¹ for an allegedly rapidly running ornithomimid. The latter estimate is questionable as there is some doubt concerning the number of prints (and thus the stride length) of the trail^{3,4}. Dinosaurs responsible for trackways in British Columbia⁵, South Wales⁶ and Queensland⁷ all seem to have been moving slowly. Thus reliable estimates of dinosaur speeds are all rather low. Here I report dinosaur speeds based on trails at a new site from the Lower Cretaceous of Texas, some of which appear quite fast by Alexander's method¹.

The site lies along Middle Copperas Creek in western Kimble County, Texas, on the F⁶ Ranch. The tracks occur in the lower third of an approximately 1-metre-thick, thin-bedded, very fine-grained limestone. Rock layers containing footprints are exposed in a narrow belt on either side of the creek bed. The limestone is heavily mud-cracked, and in some places ripple marks are preserved. The precise stratigraphical level of the locality is uncertain; the track-bearing unit is in either the lower Fort Terrett Formation or the upper Glen Rose Formation (US Gulf Coast age is Comanchean; European age equivalent of the relevant part of the Comanchean is Aptian–Albian).

All the F⁶ Ranch tracks seem to have been made by carnivorous dinosaurs (theropods) of various sizes. Trackway data are given in Table 1. A stride is taken as the distance between corresponding points on successive prints of the same foot; a pace is the distance between corresponding points on successive prints of the opposite feet, or half a stride. The larger the number of tracks in a trail, the more accurate will be the speed estimated from that trail. The estimated speeds of the F⁶ Ranch theropods range from 1.8 to 11.9 m s⁻¹. Most of the speeds are within the range reported previously, but three are considerably faster (Fig. 1). Alexander¹ indicated that mammals shift from walking to trotting or running when the ratio of stride/hip height reaches 2.0. On this basis, at least five of the F⁶ Ranch dinosaurs would have been moving with a faster gait than a walk, and the makers of trails 86→0–82, BLV→A3 and Q94→Q98 were probably running.

Alexander *et al.*⁸ described a relationship between stride length, animal size and speed, based on observations of rapidly running African ungulates, that they felt to be more appropriate for fast gaits than Alexander's earlier equation. Applying this revised equation results in slightly greater speeds for the three running dinosaurs (Table 1).

Russell⁹ suggested on anatomical grounds that ornithomimids could have reached speeds of 22.2 m s⁻¹. Applying a formula taken from Bakker¹⁰, Coombs¹¹ calculated that some ornithomimids might have attained speeds of 26.9 m s⁻¹, and that large theropods like *Allosaurus* and *Tyrannosaurus* might have had top running speeds of 12.8–14.7 m s⁻¹. However, Coombs expressed doubts as to the reliability of these estimates. Nevertheless, if these estimates of top running speed in theropods are even approximately correct, they suggest that the estimated speeds of the F⁶ Ranch running theropods are not unreasonable.

Although there is no obvious relationship between the sizes of the F⁶ Ranch dinosaurs and their speeds, only the smaller or medium-sized reptiles seem to have been running rapidly. One might expect moderate-sized dinosaurs to have reached greater speeds than their larger contemporaries^{11,12}, although this has been disputed¹³.

Coombs¹¹ concluded that the running abilities of dinosaurs were inferior to those of modern cursorial mammals, a contention that has been both supported¹² and challenged¹³ by other workers. Human athletes can achieve speeds of 10 m s⁻¹ over short distances; greyhounds and horses reach speeds of 19 m s⁻¹ in races¹⁴. In the wild, African ungulates have been clocked at speeds of 7–14 m s⁻¹ (ref. 8), and ostriches may attain speeds of 12–14 m s⁻¹ (ref. 15). Thus the fastest of the F⁶ Ranch theropods were moving at speeds greater than those reached by human athletes, but not as fast as the top speeds of modern cursorial animals. Whether the estimated speeds of the F⁶ Ranch running



Fig. 1 Three footprints of trail BLV→A3, made by one of the running dinosaurs. Pace lengths for this part of the trail are 271.8 cm (A1→A2) and 261.6 cm (A2→A3); stride length, 530.9 cm. Estimated speed of the dinosaur, 8.3 m s⁻¹.

Table 1 Estimates of dinosaur speeds at the F⁶ Ranch site

Trackway	No. of prints	Mean print length (cm)	Mean stride (cm)	Estimated speed (m s ⁻¹)	Estimated speed (km h ⁻¹)	Stride/hip height
CHHB → G48	5	47	322.6	2.6	9.4	1.7
M-73 → M-66	6	45	299.4	2.5	8.8	1.7
9B → JOF9	3	42	337.8*	3.3	11.7	2.0
K-69 → K-70	2	42	284.4†	2.5	8.9	1.7
L-71 → L-72	2	41	287.0†	2.5	9.1	1.7
18D → PVW1	5	40	296.3	2.8	10.0	1.9
86 → 0-82	7	38	658.9	11.1 (11.9)‡	39.9	4.3
N-77 → MKR32	5	38	288.3	2.8	10.0	1.9
BLV → A3	4	37	538.5	8.3 (9.4)‡	29.9	3.7
E23 → HOPE	15	37	269.1	2.6	9.5	1.8
F-90 → P-92	4	37	260.3	2.5	8.9	1.8
H-52A → H-53	3	37	322.6*	3.4	12.4	2.1
J60 → J64	7	37	214.1	1.8	6.4	1.5
C-17 → RW	3	34	259.1*	2.7	9.6	1.9
Q94 → Q98	5	29	565.6	11.9 (12.1)‡	42.8	4.9

* Estimate based on a single stride measurement. † Estimate based on twice the value of a single pace. ‡ Revised formula for rapid gaits.

theropods represent their top running speeds is, however, unknown.

I have assumed that the method of Alexander¹ accurately estimates the speeds of dinosaurian trail-makers, but this can probably never be proved. However, the relatively long strides taken by three of the F⁶ Ranch dinosaurs suggests that, unless they had unusually long legs, these reptiles were moving faster

than the makers of any other known dinosaur trails, whatever their absolute speed.

I thank Mr and Mrs David Akers, Mr and Mrs Mark Staton and Mr Louis Horner for their hospitality during my visit to the F⁶ Ranch. Dr J. Cotter Tharin and students from Hope College, Michigan, assisted with field work. Donald Baird, Paul Olsen, Philip Currie, Tony Thulborn and Walter Coombs provided helpful comments on dinosaur trackways and running abilities.

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Cytotoxic T lymphocytes induced by syngeneic mouse melanoma cells recognize human melanomas

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The recognition by T cells of antigens displayed or presented on the cell surface is generally restricted by the products of genes in the major histocompatibility complex (MHC)¹. Thus, most cytotoxic T cells recognize antigens in conjunction with self MHC determinants, especially in the response against virus-infected lymphocytes², chemically modified self antigens³ and tumour cells⁴. In these situations, the majority of cytotoxic T-cell clones seems to recognize MHC determinants unique to the individual alleles ('private'-type determinants). However, certain cell surface antigens seem not to be subject to such rigid MHC restriction^{5–7}. These apparent exceptions could be incorporated into the MHC-restriction model by postulating the involvement of public-type determinants of MHC as restriction site, but until now no cross-killing across species barrier has been reported in these instances. We now report that mouse cytotoxic T cells induced in primary culture by syngeneic melanoma cells killed various human melanoma cell lines in an antigen-specific fashion.

Cytotoxic T lymphocytes (CTL) against syngeneic malignant melanoma cells (B16) were induced in *in vitro* primary culture of naive C57BL/6 mouse spleen cells with mitomycin C(MMC)-treated B16 melanoma cells. The cytotoxic activity was melanoma specific, since the cytotoxic lymphocytes killed only syngeneic melanoma cells (B16) but not C57BL/6 lymphoma (EL-4), C57BL/10 sarcoma (S913) or A/J sarcoma (S1509a) cells. As shown in Fig. 1, the cytotoxic lymphocytes were able to lyse four different human melanoma cell lines (P-22, P-36, P-39, HMV-I) as well as mouse melanoma (B16), while they did not affect the other human cell lines tested—lymphoma (OKU) and cervix carcinoma (HeLa-S3). The cross-reactive cytotoxic activity was completely abrogated by treatment of the cells with monoclonal anti-Thy-1.2 and complement (C). This indicates that the cells responsible for the cross-reactive cytotoxicity have T-cell characteristics (Fig. 2). These results, therefore, clearly show that mouse CTL specific for syngeneic melanoma cells definitely cross-reacted with human melanoma cells, acting across a species barrier.

The cross-reactive specificity of the CTL was confirmed by using a cell inhibition assay, demonstrating that both human and mouse melanoma cells specifically inhibited CTL activity against syngeneic mouse melanoma cells (B16). In this experiment, the activity of the CTL was assayed against ⁵¹Cr-labelled B16 melanoma cells. Various unlabelled mouse and human tumour cell lines were added to the assay system as blockers with different cell numbers. The results clearly showed that both mouse and human melanoma cells (B16, P-22, P-36, P-39, HMV-I) but not other tumour cell lines (EL-4, OKU, HeLa-S3) gave strong inhibition of the CTL activity against B16 melanoma (Table 1). The inhibitory effects were specific, since the cytotoxic activity of the EL-4-specific CTL, induced in the *in*

vitro primary culture of naive C57BL/6 spleen cells together with MMC-treated EL-4, was blocked by EL-4, but *not* by the mouse and human melanoma cells which inhibited melanoma-specific CTL (Table 1). Also, the possible involvement of the fetal calf serum component of the culture media as acquired determinants on the target cell surface was excluded, since the cytotoxic activity was observed on and inhibited only by melanoma cell lines from both human and mouse origins and not by other human and mouse tumour cell lines.

Thus our results show that malignant melanoma cells carry specific antigenic determinants shared with both mouse and human species—that is, melanoma-specific antigens have common antigenic moieties in different animal species. This is also supported by our finding that cytotoxic antisera raised in C57BL/6 mice hyperimmunized with MMC-treated B16 melanoma cells could specifically react with both mouse and human melanoma cell lines but not other cell lines (data not shown).

In general, the identity of the histocompatibility between T cells and their target cells is necessary for the cytotoxic T-cell

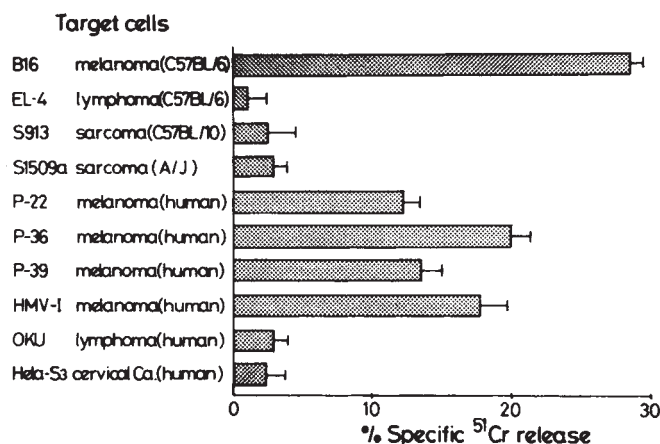


Fig. 1 Cross-reactive cytotoxic effect of mouse melanoma (B16)-induced syngeneic (C57BL/6) killer cells on human melanomas. C57BL/6 cytotoxic lymphocytes (CTL) against syngeneic mouse melanoma (B16) cells were induced in the *in vitro* primary response. In brief, 3×10^7 spleen cells from naive C57BL/6 mice were cultured alone as a control or with 6×10^5 of MMC-treated B16 melanoma cells in 3 ml Dulbecco's modified minimal essential medium (DMEM) containing 5% fetal calf serum (FCS) and 5×10^{-5} M 2-mercaptoethanol in 3.5 cm $\times$ 1 cm Petri dish (Falcon) at 37 °C in humidified 5% CO₂ in air. Five days later, the cells were pelleted by centrifugation and adjusted to 6×10^6 viable nucleated cells per ml. To assay the activity of the cultured cells, the lymphoid cells (6×10^5) were mixed with 1.5×10^4 ⁵¹Cr-labelled target cells (target/CTL ratio 1:40) in 0.2 ml of DMEM in a multi-well plate (NUNC) in quadruplicate. Target cells were mouse and human cell lines: C57BL/6 melanoma, B16; C57BL/6 lymphoma, EL-4; C57BL/10 fibrosarcoma, S913; A/J fibrosarcoma, S1509a; human melanoma cell lines, SK-MEL-26(P-22), SK-MEL-28(P-36), MeWo(P-39), HMV-1; human lymphoma, OKU; and human carcinoma, HeLa-S3. The plates were incubated for 12 h at 37 °C and the radioactivity in a 0.1 ml supernatant from each well was counted by a well-type γ scintillation counter (Dainabot). Per cent lysis was expressed as percentage of specific ⁵¹Cr release calculated by the formula:

% Specific ⁵¹Cr release

$$= \frac{\text{c.p.m. experimental release} - \text{c.p.m. control release}}{\text{c.p.m. maximum release} - \text{c.p.m. control release}} \times 100$$

Control release was determined by incubating ⁵¹Cr-labelled target cells with naive spleen cells of C57BL/6 mice cultured for 5 days without stimulator cells (B16). Maximum lysis was obtained by disrupting the target cells with saponin (Wako Pure Chemical). The bars indicate arithmetic means of the per cent specific lysis for four cultures $\pm$ s.d.

interaction¹. In these studies, T cells have been shown to recognize MHC-encoded products, especially determinants that are highly polymorphic within species and those closely related to the serologically defined histocompatibility antigens. Thus, the requirement for T-cell recognition of MHC determinants has been demonstrated on the basis of MHC restriction of the cytotoxic activity. However, there are a few reports that MHC identity is not required for the interaction between cytotoxic T cells and allogeneic target cells⁸⁻¹⁰. For example, Burton *et al.*⁸ have described the primary *in vitro* responses of murine cytotoxic T cells specific for oncofetal and plasmacytoma antigens that do not appear to be H-2 restricted. Similarly, Shaw *et al.*⁹ have demonstrated that the human cytotoxic T cell induced by trinitrophenol (TNP)-modified autologous cells was not restricted to lysis of TNP-modified human target cells without carrying shared HLA determinants with targets, and that the human CTL raised against TNP-self was clearly restricted with respect to lysis of mouse target cells. Nevertheless, these apparent exceptions could be explained by the MHC-restriction model that T cells recognize antigens in association with MHC determinants being widely shared within species.

However, the results presented have failed to show MHC restriction, and CTL specific for syngeneic mouse melanoma killed human melanoma cell lines across a species barrier. The products of genes in the I-E/I-C but not I-A subregion of the mouse H-2 complex share interspecies cross-reactive determinants with human HLA-DR antigens^{11,12}, and human melanoma cell lines have also been reported to express HLA-DR like antigens^{13,14}. Thus the cross-reactive DR or Ia antigens are possible candidates to serve as 'self' determinants recognized by CTL. However, C57BL/6 mice as used in our studies have been shown not to express I-E/I-C subregion gene products¹¹, so the B16 melanoma of C57BL/6 origin seems not to express cross-reactive Ia antigens on the cell surface. It is likely, therefore, that the CTL recognizes the melanoma-specific antigen itself, rather than an association of the antigen with MHC determinants. If so, the MHC restriction in the T-cell response may be determined by the nature of antigens with or without MHC products. If, however, common MHC determinants are conserved in different animal species (at least between mouse and man), it is also possible that mouse CTL recognizes melanoma-specific antigenic determinants in association with such conserved MHC determinants on human melanoma cells. We cannot yet determine whether one or more of these alternatives operate in tumour immunity, and experiments are under way to look into this question.

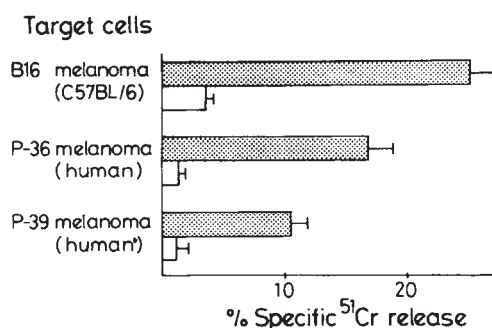


Fig. 2 Effect of the treatment with anti-Thy-1.2 on the cytotoxic activity of B16-induced syngeneic (C57BL/6) killer cells against mouse and human melanomas. The C57BL/6 CTL induced by syngeneic mouse melanoma cells (B16) as described in Fig. 1 was treated with a 1:1,000 dilution of monoclonal anti-Thy-1.2 (F7D5) for 30 min at 37 °C followed by incubation with well selected rabbit complement at 37 °C for 45 min. The activity of CTL treated was assayed by the same method as described in Fig. 1 legend. The bars indicate arithmetic means of the specific lysis of four cultures $\pm$ s.d. Solid and open bars indicate the activity of the CTL treated with complement alone or that treated with anti-Thy-1.2 and complement, respectively.

Table 1 Specific inhibitory effects of human melanoma cells on the cytotoxic activity of C57BL/6 killer T cells specific for B16 melanoma cells

Inhibitor cells	Labelled/unlabelled cell ratio	% Specific ⁵¹ Cr release	
		C57BL/6 anti-B16 on B16 melanoma	C57BL/6 anti-EL-4 on EL-4 lymphoma
None		23.9 ± 1.2	34.3 ± 2.2
B16 melanoma (C57BL/6)	1:9	6.3 ± 0.6	32.8 ± 2.1
EL-4 lymphoma (C57BL/6)	1:9	24.1 ± 1.2	9.0 ± 1.4
P-22 melanoma (human)	1:9	3.2 ± 1.2	ND
P-36 melanoma (human)	1:9	5.0 ± 1.4	26.6 ± 2.1
P-39 melanoma (human)	1:9	7.2 ± 1.0	27.5 ± 2.0
HMV-I melanoma (human)	1:9	2.1 ± 1.0	ND
OKU lymphoma (human)	1:9	22.4 ± 0.3	ND
HeLa-S3 carcinoma (human)	1:9	20.4 ± 0.9	ND

The activity of the C57BL/6 CTL-induced B16 melanoma or EL-4 lymphoma was assayed as described in Fig. 1 legend on ⁵¹Cr-labelled B16 or EL-4 tumour target cells (target/CTL ratio 1:40), respectively. Various unlabelled mouse and human tumour cell lines were added to the assay system as blockers with different cell numbers (labelled/unlabelled = 1:0, 1:1, 1:3, 1:9). The assay conditions and tumour cell lines were the same as described in Fig. 1 legend. The absolute number of the CTL and ⁵¹Cr-labelled B16 melanoma or EL-4 lymphoma cells as targets remained constant (target/CTL ratio of 1:40). Only blocker cell number was varied. Results are expressed as arithmetic means of the specific lysis of four cultures ± s.d.; ND, not determined.

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Antigen-driven helper cell-independent cloned cytolytic T lymphocytes

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Cellular interactions between alloreactive cytolytic T lymphocytes (CTLs) and helper T cells have been suggested by the results of experiments in which lymphocyte subpopulations were defined by antisera against cell-surface markers¹ or by differential response to antigenic stimulation^{2,3}. The development of T-cell cloning technology has allowed the isolation of alloreactive cloned helper and cytolytic cells, and the demonstration of collaboration between them in the generation of cytotoxicity^{4,5}. Whereas some types of alloantigen-specific cloned T cells, including helper cells, proliferate in tissue culture in response to antigenic stimulation⁴⁻⁶, CTLs isolated thus far do not and require for proliferation the addition of exogenous T-cell growth factors (TCGFs) or helper cells which produce TCGF in response to antigenic stimulation^{4,5,7-10}. We demonstrate here, at the clonal level, the existence of another type of CTL which proliferates in response to allogeneic stimulating cells without the overt addition of TCGF; it produces helper-like factor and thus can be characterized as a helper cell-independent CTL.

T-cell clones were derived by limiting dilution from 5-day C57BL/6 (B6) anti-DBA/2 (DBA) bulk mixed lymphocyte culture (MLC) populations as previously described⁷. Limiting dilution microcultures for cloning were seeded on day 5 of MLC with 0.25 B6 anti-DBA cells per well, 1×10^6 irradiated (2,000 rad) DBA spleen cells and 30% (v/v) secondary MLC

supernatant (2° MLC SN)¹¹ as a source of growth factors, including TCGF. After 7-8 days, growing clones were further expanded numerically in larger culture vessels in the presence of DBA spleen cells and 20% 2° MLC SN and maintained by subculturing at 5-7-day intervals. Such cloned populations were tested repeatedly for cytolytic activity against P815 (DBA origin) and EL4 (B6 origin) tumour target cells in both direct and lectin-dependent ⁵¹Cr release assays and for their ability to proliferate in response to allogeneic DBA stimulating cells.

Table 1 shows the proliferative responses of two cloned CTL populations and two cloned helper populations. Clones 17-11 and 17-4 are CTLs which cause 50% ⁵¹Cr release from P815 target cells in a direct test at effector/target ratios of 1:1 to 3:1; no detectable lysis of EL4 cells is observed at ratios of up to 30:1. Clones 12-11 and 17-10 can be characterized as non-cytolytic cells based on their inability to kill P815 or EL4, even in the presence of lectin, and as helper cells based on their ability to produce soluble factor(s) capable of stimulating proliferation in a TCGF assay using long-term (days 14-18), resting, primary MLC populations as indicator cells (unpublished observation).

All clones proliferated in culture when 2° MLC SN was present, regardless of whether splenic filler cells were also present (Table 1). However, in the absence of exogenous 2° MLC SN, only certain clones, such as helper cell clones 12-11 and 17-10, proliferated in response to specific allogeneic DBA stimulating cells. Cultures of CTL clone 17-11 and allogeneic DBA stimulating cells did not incorporate amounts of ³H-thymidine (TdR, 3,066 c.p.m.) greater than those incorporated in control cultures using syngeneic B6 stimulators (1,048 c.p.m.) or stimulating cells alone (3,736 c.p.m.) (Table 1). This CTL clone thus exhibits the growth requirement for TCGF typical of CTL clones previously described^{4,5,7-10}; in our experience, six B6 anti-DBA CTL clones behaved in the proliferative assay like CTL clone 17-11. In contrast, CTL clone 17-4 demonstrates the distinct ability to proliferate in response to allogeneic DBA stimulating cells, even in the absence of 2° MLC SN (23,792 c.p.m.).

Table 1 Proliferative response of T-cell clones

Responding cell clone	2° MLC SN	Stimulating cells		
		None	C57BL/6 _x	DBA/2 _x
17-11	—	490 ± 240*	1,048 ± 242	3,066 ± 278
	+	11,808 ± 776	10,194 ± 460	41,628 ± 3,566
17-4	—	1,084 ± 300	1,348 ± 124	23,792 ± 298
	+	80,432 ± 5,380	64,296 ± 912	48,270 ± 3,816
12-11	—	1,764 ± 1,348	2,554 ± 1,090	42,200 ± 958
	+	28,914 ± 2,614	30,424 ± 1,864	39,660 ± 1,032
17-10	—	904 ± 608	1,512 ± 406	18,192 ± 602
	+	21,660 ± 2,200	14,194 ± 376	25,174 ± 1,598
None	—	NT	1,346 ± 888	3,736 ± 2,482
	+	NT	1,264 ± 146	3,690 ± 130

Proliferative response of alloreactive clones in MLC. Clones were derived by limiting dilution from 5-day B6 anti-DBA bulk MLC populations⁷. Cloned cells were expanded and maintained by subculturing $1-5 \times 10^4$ cloned cells at 5–7-day intervals in 16-mm Costar wells containing 20% v/v 2° MLC SN and 6×10^6 irradiated (2,000 rad) DBA spleen cells^{5,7}. The culture medium consisted of Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum and additional amino acids¹³. For the proliferative assay, 1×10^4 cloned cells obtained 5–7 days after previous subculture were cultured (0.2 ml final volume) in flat-bottom microtitre plates either alone or with 1×10^6 irradiated syngeneic B6 or allogeneic DBA spleen cells in the presence or absence of 25% 2° MLC SN. Cultures were pulsed on day 2 for 7 h with 2 μ Ci ³H-TdR. Cells were collected on to glass fibre filters and ³H-TdR incorporation was determined by liquid scintillation spectrometry. Results are given as mean c.p.m. ³H-TdR incorporated $\pm$ s.d. of triplicate cultures. NT, not tested.

Because cells of putative clone 17-4 exhibited the lytic characteristics of CTLs and the proliferative characteristics of T-helper cells, we investigated whether both functions were in fact mediated by the progeny of a single cell, rather than two functionally distinct cells contained within the 17-4 population, by analysing subclones derived from 17-4 cells in both the cytolytic and proliferative assays. Subcloning was accomplished by plating 17-4 cells in limiting dilution microcultures at a concentration of 0.1 cells per well. Of 168 microcultures established, 20 were observed to contain growing cells when examined microscopically 7 days after culture initiation. Thus, cells from putative clone 17-4 had 100% plating efficiency (16 positive microcultures were expected based on the Poisson distribution). Cells from five positive subcloning cultures were picked, expanded numerically and tested for both cytolytic activity against P815 and proliferation in response to irradiated DBA spleen cells. Figure 1 demonstrates that cells of parent clone 17-4 and all five tested subclones were highly cytolytic towards P815 target cells, causing 50% ⁵¹Cr release at effector/target ratios between 1.8:1 and 3:1. In addition, cells

of all five 17-4 subclones were, like those of the 17-4 parent clone, able to proliferate in response to irradiated DBA spleen cells in the absence of overtly added 2° MLC SN (Table 2). The probability that any one of these subclones could have been derived by chance alone from more than one cell in the parental population of putative clone 17-4, given the 0.1 cell per well dilution used for subcloning, is 5×10^{-2} ; the probability for all five subclones is 3×10^{-7} . Thus, there can be no reasonable doubt that both cytolytic activity and proliferative response to allogeneic cells are in this case functions of the progeny of a single T cell. Karyotypic analysis of 17-4 cells has shown them to have the normal chromosome number of $2n = 40$.

The proliferative response of cells of clone 17-4 in the presence of allogeneic stimulating cells could conceivably be induced by factors secreted by cells, presumably T cells, within the irradiated stimulating cell population, which recognize the cloned cells as foreign—that is, by 'back stimulation'. However, the data in Table 3 demonstrate that allogeneic cells depleted of T cells by pretreatment with anti-Thy-1.2 antibody and complement stimulate cells of subclone 17-4.24 to proliferate,

Table 2 Proliferative response of clone 17-4 and its subclones

Responding cell clone	2° MLC SN	Stimulating cells		
		None	C57BL/6 _x	DBA/2 _x
17-4	—	1,038 ± 563*	1,628 ± 428	24,178 ± 800
	+	34,303 ± 1,446	19,932 ± 319	21,079 ± 1,920
17-4.21	—	981 ± 505	2,136 ± 361	32,506 ± 1,109
	+	72,557 ± 5,457	45,103 ± 7,873	34,318 ± 1,530
17-4.22	—	616 ± 306	638 ± 18	12,220 ± 483
	+	23,316 ± 1,256	19,998 ± 915	26,654 ± 577
17-4.23	—	2,866 ± 902	1,734 ± 574	20,315 ± 2,689
	+	61,109 ± 3,340	36,086 ± 2,019	30,353 ± 1,049
17-4.24	—	439 ± 294	1,297 ± 1,025	42,153 ± 3,249
	+	83,044 ± 814	40,846 ± 3,232	40,634 ± 5,706
17-4.28	—	309 ± 278	898 ± 332	14,102 ± 2,614
	+	48,593 ± 1,819	31,956 ± 3,678	41,366 ± 1,003
None	—	NT	1,152 ± 641	1,328 ± 428
	+	NT	1,274 ± 394	2,752 ± 799

Proliferative response in MLC of CTL clone 17-4 and its subclones. Subclones were derived by plating 17-4 cells at a concentration of 0.1 cells per well in the presence of 1×10^6 irradiated DBA spleen cells and 30% 2° MLC SN. Cloned cells were expanded, maintained and tested for proliferative responses in MLC as described in Table 1 legend.

Table 3 Proliferative response of cloned cells to T-cell-depleted stimulating cells

Responding cell clone	Treatment of stimulating cell population	Stimulating cells		
		None	C57BL/6 _x	DBA/2 _x
17-4.24	C'	276 ± 80*	796 ± 300	4,505 ± 548
	Anti-Thy-1.2 + C'		548 ± 262	6,215 ± 330
None	C'	NT	561 ± 125	553 ± 102
	Anti-Thy-1.2 + C'		288 ± 35	505 ± 71

Proliferative response of subclone 17-4.24 to T cell-depleted stimulating cells. Cloned cells were tested for proliferation in response to allogeneic spleen cells in the absence of 2° MLC SN as indicated in Table 1. Syngeneic B6 or allogeneic DBA spleen cells were incubated at room temperature for 30 min with a 1:1,000 dilution of monoclonal anti-Thy-1.2 antibody (NEN), followed by incubation at 37 °C for 45 min with a 1:10 dilution of rabbit complement (C'). Control aliquots were incubated with complement alone. Antibody and control-treated cells were washed three times, irradiated (2,000 rad) and used as stimulating cells (1×10^6 cells per culture) in MLC with cloned responding cells.

thus eliminating back stimulation as the sole or primary explanation for the proliferative response. In addition, specificity analyses using congenic H-2 recombinant strains have shown specificity of clone 17-4 in both the proliferative and cytotoxic assays against D^d-encoded antigens; no proliferative response (and thus no back stimulation that might be expected) is observed even when cells from strains bearing entirely different H-2 antigens (H-2^s and H-2^k) are used as potential stimulators of 17-4 cells.

We have shown here, on the clonal level, that alloreactive CTLs can be divided into at least two distinct categories based on their requirements for proliferation. The first is the 'conventional' CTL represented by our clone 17-11 and also isolated by others^{4,5,7-9}. We present evidence here for the existence of a second type of CTL, exemplified by clone 17-4. In contrast to cells of the first category, this cell does not require independently derived sources of helper factor for proliferation, as shown by its ability to proliferate in response to stimulating cells bearing the sensitizing alloantigens. In this respect clone 17-4 is a helper cell-independent CTL clone, and represents, to our knowledge, the first proven clone with such characteristics to be reported.

We emphasize that clone 17-4 is the only CTL clone of the seven we have tested to date which exhibits responsiveness in MLC to allogeneic cells. Thus, the frequency of such cells in bulk MLC populations from which the clones are derived (or the frequency of detection) seems to be relatively low. However, the existence of this type of cell has recently been confirmed both independently (A. Glasebrook, personal communication) and in our own laboratory (D. Roopenian, unpublished observation).

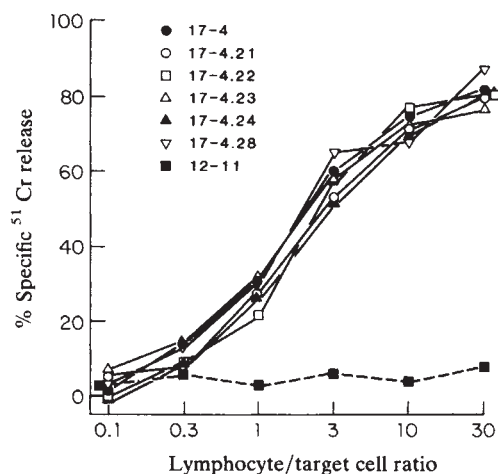


Fig. 1 Cytolytic activity of clone 17-4 and its subclones. Cells from clone 17-4 and five subclones (solid lines) and helper cell clone 12-11 (dashed line) were tested at the indicated effector cell/target cell ratios for cytolytic activity against 2×10^3 ^{51}Cr -labelled P815 target cells in a 3.5-h assay¹⁴. Cells used for cytotoxicity testing were obtained 5 days after subculture.

Glasebrook and Fitch have reported that helper cell clones can utilize for proliferation growth factor(s) which they themselves produce¹². Preliminary evidence suggests that cells of CTL clone 17-4, like cloned helper cells, secrete a soluble factor when stimulated with allogeneic cells which induces proliferation and cytolytic activity in resting, long-term (day 14) MLC populations. Whether the factor produced by clone 17-4 is the same as that (those) produced by the conventional type of non-cytolytic helper cell is not known, but such a factor could be involved with antigen in the induction of proliferation by the clone. We thus propose that clone 17-4 has both helper and CTL functions previously considered unique to different T-cell subsets. Such attributes would presumably constitute an effector T cell that depends only on antigen for expansion. Whether this type of cell represents a stage in the T-cell lineage before or after differentiation of cells exhibiting mutually exclusive functions remains unknown.

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Inward current channels activated by intracellular Ca in cultured cardiac cells

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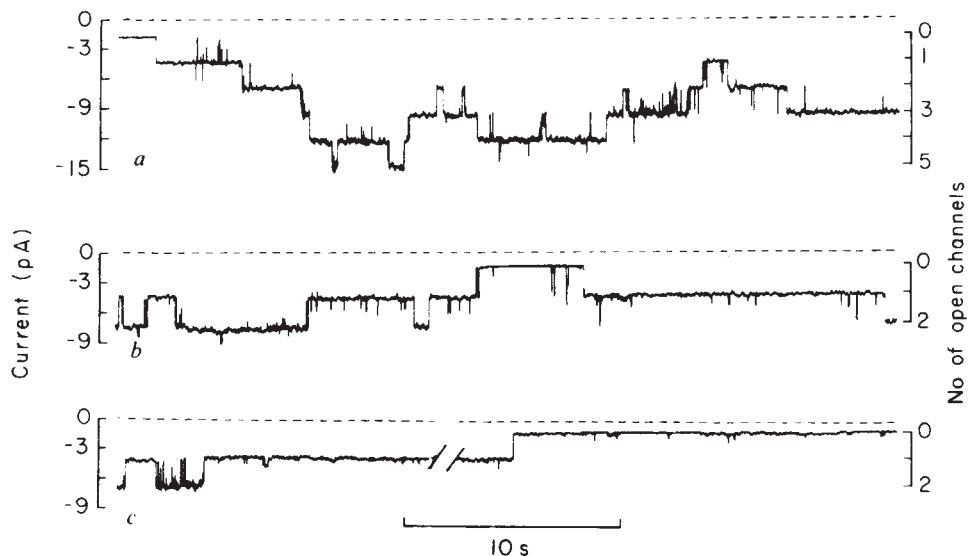
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Present concepts of excitable membrane function are based primarily on knowledge of two classes of channels: those activated by neurotransmitters¹ and those activated by membrane potential². Recent evidence suggests that these notions may have to be modified to include other channel types, such as special ion channels activated by membrane potential but regulated by ligands³⁻⁵. We report here studies on single channel currents recorded from heart muscle cells, in which we have found a channel, abundant in cardiac membrane, which does not seem to belong in any of the familiar categories. This channel shows little selectivity between cations, but excludes anions. It is activated by intracellular Ca ions but is not appreciably affected by membrane potential.

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Fig. 1 Recordings of single-ion channel currents at three Ca concentrations from inside-out membrane patch of cultured (day 4) rat ventricular muscle cell at 26°C. The pipette (external side of membrane) contained Na-saline with 6 μ M Ca. The broken line above each trace marks zero current; inward current is shown as a downward deflection. The membrane patch was voltage-clamped to -70 mV, which is seen to require ~1.5 pA (patch resistance ~50 G Ω) when all channels are closed. The unit current is ~2.7 pA (conductance 38 pS; in *b* and *c* two smaller inward current steps are clearly resolved). *a*, Bathing solution (cytoplasmic side of membrane) of Na-saline with 6 μ M Ca (recording started soon after patch isolation). *b*, Bathing solution of K-saline with 1.5 μ M Ca (flow started 11 s before start of record). *c*, Bathing solution of 'Ca-free' saline + EGTA (10 μ M). Flow started a few seconds before start of record. After brief flickering, one channel remained open for 35 s before closing (the break in the record denotes a 22-s gap throughout which this channel alone remained open); then all channels remained closed while EGTA was still present in the solution. Re-addition of calcium (not shown) caused the channels to open again. Note the quiet baseline in *a* and *b* when all channels were closed and the increase in 'noise' when the channels were open.



In our experiments we used myocytes obtained from neonatal rat hearts and cultured by a method similar to that described elsewhere⁶. The cells were exposed to trypsin (0.025%) for 24 h before seeding on day 0 and used before day 7. We selected for our experiments single cells having spindle-like shapes and striations. Single-channel currents were recorded from isolated patches of membrane with either the cytoplasmic surface (inside-out patch) or the external surface (outside-out patch) facing the bathing solution⁷. The Na-saline solution contained (mM): NaCl, 137; KCl, 5.4; MgCl₂, 1.05; HEPES buffer, 5.0; glucose, 10; pH 7.3. In the K-saline solution, NaCl was replaced by KCl. CaCl₂ was added, and the final Ca concentration determined by atomic absorption spectroscopy.

Channel currents of the type shown in Fig. 1 were seen in most isolated membrane patches. We have interpreted such records as arising from the superimposed currents of several independent channels that can only open and close, but we cannot exclude the possibility that individual channels have multiple equally spaced conductance levels. The rate of channel opening was clearly dependent on the Ca concentration on the cytoplasmic side of the membrane (see Fig. 1). Considerable channel activity was usually observed in the presence of 6 μ M Ca (Fig. 1*a*). After reduction of the Ca concentration to 1 or 1.5 μ M, the activity decreased (Fig. 1*b*) but was still substantial. In the presence of 'Ca-free' saline + EGTA (10 μ M), all channels eventually closed (Fig. 1*c*). In several experiments it was possible to change from Ca-containing solution to EGTA-saline and back several times, and to observe repeatedly the disappearance and reappearance of the single-channel currents. The assessment of the Ca-concentration dependence of channel opening was complicated by the fact that even when the Ca concentration was kept constant, the channel opening rate was usually observed to decline over a period of several minutes. A subsequent increase in activity was often seen when the Ca concentration was raised (for example, to 20 or 50 μ M). However, changes in Ca concentrations over a wide range did not affect single-channel conductance. In many experiments similar channel currents were observed when the patch clamp pipette was applied to the intact cell, without removal of the membrane patch; in these cases no obvious contracture of the cell was observed.

The opening and closing of the Ca-dependent channels did not show any strong dependence on membrane potential at a Ca concentration of 20 μ M. Single-channel currents occurred with

similar frequencies at membrane potentials from -140 mV to +70 mV. In other experiments, records were obtained while stepping the membrane potential either from -70 mV to +70 mV for 100 ms, or from -70 or -120 mV to -30 mV for 5 s. When a series of such records is averaged, if the number of open channels were voltage-dependent it would be expected that the mean current would show a relaxation towards a new equilibrium value following a step in membrane potential. Such relaxations were observed, but they were very small, indicating that the open-close equilibrium changes less than *e*-fold for 200 mV. We cannot exclude the possibility that stronger voltage dependence might be present in other conditions.

The opening and closing patterns of these channels are rather complex. In conditions of low activity when at most one channel

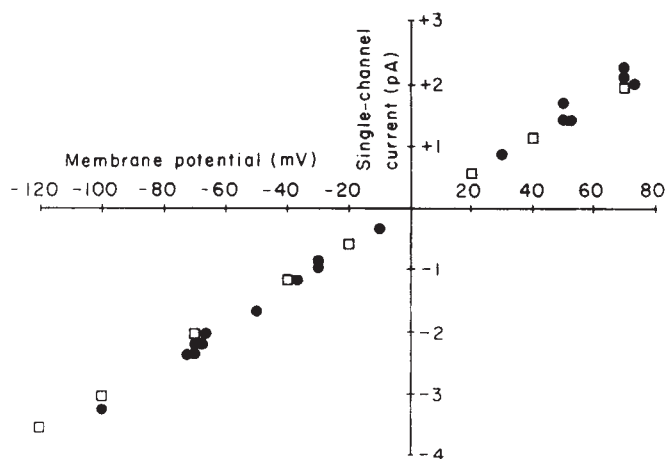


Fig. 2 Relationship between amplitude of single-channel current and membrane potential, with symmetrical and asymmetrical solutions at 25–26°C. Values at positive potentials were obtained by short (100 or 200 ms) jumps from the holding potential (-70 mV) to a positive potential. All measurements were made at 10-mV intervals, but, when necessary for clarity, the symbols have been slightly offset horizontally. ●, Na-saline with 20 μ M Ca on both sides of the membrane; values from five different cells (inside-out membrane patches) on day 6 of culture. □, K-saline with 1 μ M Ca on cytoplasmic side of membrane, and Na-saline with 2 mM Ca on the external side of the membrane (outside-out membrane patch); cells were used on day 2 of culture.

was open, the mean open lifetime was about equal to its s.d. with values of ~100–300 ms. The shorter openings and poorly resolved spikes (see Fig. 1) represent channel lifetimes of this order. In most cases there were much longer channel openings of many seconds duration (Fig. 1). Often, for example, one channel appeared to be 'permanently' open and the resulting current formed a baseline on which the faster activity described above was superimposed. One channel seemed to remain open for ~35 s after addition of EGTA, before it eventually closed (see Fig. 1c). Even in patches where we had evidence for the presence of multiple channels, long periods occurred when one of the channels opened with a high probability and the other channels appeared not to function. This suggests that channels can also enter a long-lived closed state.

Conductance through the ion channel was approximately ohmic, as shown in Fig. 2 where the single-channel conductance was ~30 pS at 25 °C. Typically, single-channel conductances in over 50 patches ranged between 30 and 40 pS at 25–27 °C. Figure 2 shows that with identical saline on both sides of the membrane, the reversal potential for the channel current is close to the expected value of 0 mV. When all NaCl was replaced by KCl on the cytoplasmic surface (Fig. 2, □) the reversal potential was unchanged. Thus the ion channel must have similar permeabilities to Na and K ions. In another experiment we applied a salt gradient across the membrane by diluting the pipette saline to 1/5 with distilled water. Single-channel currents recorded in this condition reversed sign at -40 mV, as expected for cation selectivity. In addition, when chloride was replaced by the presumably less permeant anion, aspartate, the reversal potential did not change. These results indicate negligible anion permeability of the ion channels. The temperature dependence of the unit current (Fig. 3) has a Q_{10} of ~2.3 (two experiments), which is unusually large; for example, a Q_{10} of 1.2–1.3 has been reported for ACh-activated channels^{8,9}.

In addition to the predominant channels described above, various other channel types were seen, including a number of small inward channel currents present at -70 mV (see Fig. 1b, c) and occasional Ca-dependent inward currents that were unusually large (50–60 pS). Voltage-dependent Na, Ca and K channels were also observed.

The ion channels described here resemble ACh-activated endplate channels in their conductance, and in their poor discrimination between Na and K ions, as well as in their low permeability to Cl ions¹⁰ but their conductances are more temperature sensitive, have much longer open lifetimes, are less voltage dependent and are unaffected by carbachol. They resemble Ca-activated K channels^{11,12} in that they are activated by Ca on the cytoplasmic side of the membrane, and in their

complex kinetics. However, they seem to require higher concentrations of Ca for activation, are not so voltage-dependent and lack the cation selectivity of Ca-activated K channels.

Several authors^{13–15} have described oscillatory inward currents that occur in cardiac Purkinje fibres after depolarizing clamp steps during ouabain exposure or with high extracellular Ca concentration. This transient inward current has many features in common with the ion channels described here. It is activated by intracellular Ca ions and not by voltage directly and has a reversal potential around -5 mV indicating poor selectivity between Na and K ions¹⁴. Kass *et al.*¹⁴ speculate that the underlying mechanism for the transient inward current could be non-selective 'leak' channels which, in normal conditions, would provide the background current required for pacemaking activity in cardiac cells. This hypothesis would be consistent with our results.

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Free Ca^{2+} increases in exponential phases during mouse oocyte activation

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A dramatic rise in cytoplasmic free Ca^{2+} concentration has been shown to occur during fertilization and artificial activation in the oocytes of both the medaka fish¹ and sea urchin². Indirect evidence has implicated Ca^{2+} in the parthenogenetic activation of mammalian oocytes. Mouse oocytes can be activated by the intracellular injection of Ca^{2+} but not Mg^{2+} (ref. 3), and hamster oocytes by exposure to the calcium ionophore, A23187 (ref. 4). We report here measurements of cytoplasmic free Ca^{2+} during the artificial activation and fertilization of single mouse oocytes injected with the Ca^{2+} -sensitive photoprotein aequorin. Free Ca^{2+} rises exponentially from a resting level of below 0.1 μM to >5 μM over a period of 10–30 min. A series of oscillatory Ca^{2+} transients precedes this Ca^{2+} rise during fertilization, but not during artificial activation.

The light from a single 'resting', unfertilized oocyte (70 μm diameter, 200 pl volume), injected with aequorin to ~5% of its volume, ranged typically between 1 and 16 photoelectrons per s above a background of 14 s⁻¹ (Fig. 1A, B). The light signal was detected by holding the oocyte in a 75- μl -capacity reflecting cup positioned just below the photocathode of a low-noise photo-

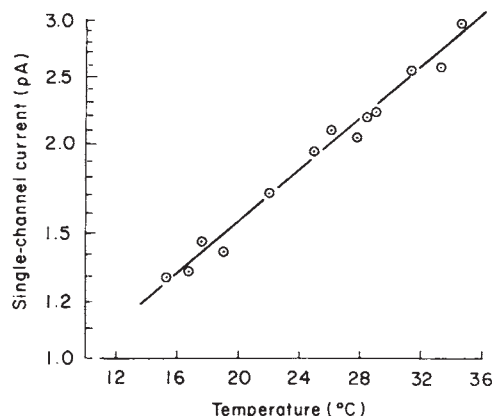


Fig. 3 Temperature dependence of the amplitude of the single-channel current. The ordinate is a logarithmic scale. Cells were used on day 2. Inside-out membrane patch with K-saline + 20 μM Ca in the pipette and Na-saline + 20 μM Ca in the bathing solution; membrane potential -70 mV.

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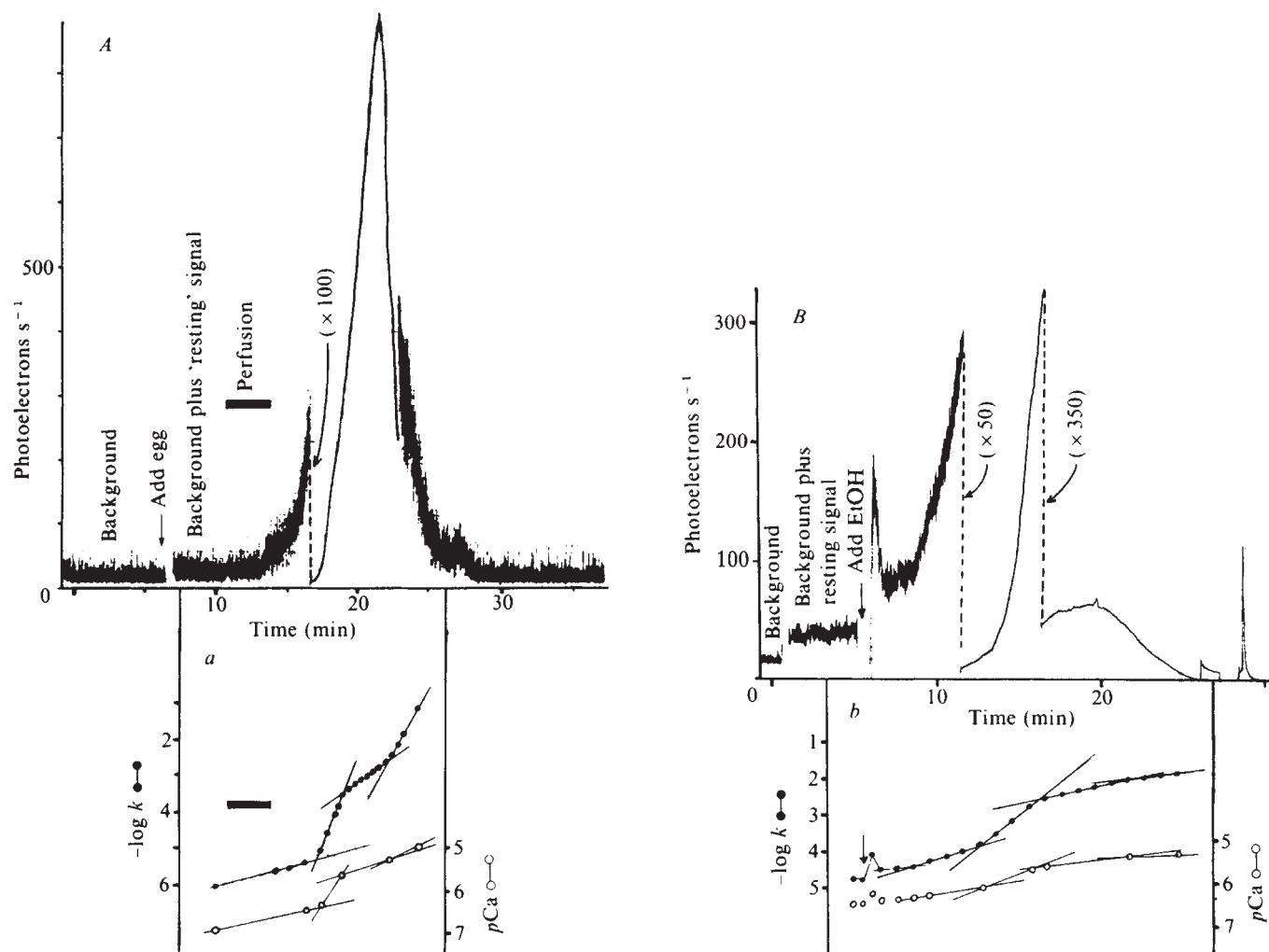


Fig. 1 *A, a*, Effect of an activating concentration of benzyl alcohol (0.5% v/v) on an aequorin-injected oocyte. *A*, Chart recording of the aequorin signal, expressed as photoelectrons s^{-1} against time. The resting signal of 6 photoelectrons s^{-1} remained constant until medium containing 0.5% benzyl alcohol was superfused (solid bar; the egg was exposed to benzyl alcohol up to the end of the experiment). Immediately the signal rose rapidly. The sensitivity of the recorder was reduced by a factor of 100 between 17 and 23 min. The signal returned to background level as the aequorin was consumed. In some experiments total consumption of aequorin was confirmed by adding SDS or distilled water to lyse the oocyte. *a*, The same data transformed to $-\log k$ (●) against time, where k is the fraction of aequorin consumed per s (that is, the ratio of the light signal in photoelectrons s^{-1} to the total number of photoelectrons yet to come from the active aequorin remaining in the oocyte). Note the piece-wise linearity of this plot, which implies that $[Ca^{2+}]_i$ increases in exponential phases. The plot is also shown in terms of pCa units (○). *B, b*, Effect of an activating concentration of ethanol (8.6% v/v) on an aequorin-injected oocyte. *B*, Chart recording of the aequorin signal in photoelectrons s^{-1} against time. Recorder sensitivity was reduced 50-fold and 350-fold at 12 and 17 min. Ten microlitres of medium with ethanol (30% v/v) were added to 25 μ l of medium in the reflecting cup containing the oocyte. The trace was blanked out during this operation. *b*, The same data plotted as $-\log k$ (●) and pCa (○) against time. Calibration measurements were made *in vitro* at 35 °C with EGTA Ca^{2+} buffers containing (mM) KCl, 150; PIPES, 10; EGTA, 10; free Mg^{2+} , 5 at pH 7.4. The stability constants of Ca -EGTA and Mg -EGTA, corrected for 37 °C and pH 7.4, were calculated at $10^{7.44}$ and $10^{2.46} M^{-1}$ respectively¹⁴. Our calibration curve of $-\log k$ against pCa is linear (with slope 3.0) from $-\log k = 5.5$ to 2.0. (Benzyl alcohol and ethanol have a direct effect on the emission of light by aequorin, as with other anaesthetics¹⁵. Benzyl alcohol (0.5%) raised k by <0.1 log unit, but ethanol (8%) raised k by 0.8 log unit (P.H.C., unpublished results). This accounts for the initial jump in $\log k$ seen in the ethanol experiment (arrow). Quantum yield is not affected.) Oocytes were collected from superovulated random-bred albino females (MF1; Olac, Bicester) and maintained at 37 °C in a Krebs-Ringer's bicarbonate-buffered medium (No. 16; ref. 16) gassed with 5% CO_2 , 95% air. Cumulus cells were removed with hyaluronidase in medium 16. Oocytes for the fertilization experiments were treated, after injection, with 0.03% α -chymotrypsin (type II; Sigma) to remove the zona pellucida. Oocytes were injected at room temperature in medium M2 (ref. 3), which is medium 16 modified by partly replacing the bicarbonate buffer with HEPES buffer. The aequorin was dialysed against an injection buffer containing 30, 80 or 150 mM KCl, 10 μ M 3-(*N*-morpholino)propanesulphonic acid (MOPS) and 25 μ M EDTA, pH 9.0. Micropipettes with a tip diameter of $\sim 2 \mu$ m were pressure-filled (at 7 bar) through the tip⁵ with the aequorin solution, which was injected by pulses of gas pressure (up to 2 bar) into an oocyte held on a siliconized heat-polished pipette. The oocyte was contained in a minimal bubble of medium at the end of the holding pipette, surrounded by silicone oil (M100; Bayer, Leverkusen). The aequorin was thus protected from the Ca^{2+} in the medium before penetration of the oocyte. All Ca^{2+} measurements described were done in medium 16 at 35 °C, except for the two experiments shown here, in which medium M2 was used. Oocytes were between 15 and 22 h post-HCG (human chorionic gonadotropin) at the time of the experiments.

multiplier. Detection limits proved to be better than predicted⁵. Assuming homogeneous Ca^{2+} distribution, and assuming that the conditions *in vivo* can be sufficiently matched *in vitro*, the rate of aequorin consumption, k , can be calibrated^{6,7} for a given Mg^{2+} concentration, to give the free intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$. The mean value of k measured in 21 'resting' oocytes at 35 °C corresponds to values for $[Ca^{2+}]_i$ of $5 \pm 1 \times 10^{-8} M$ if $[Mg^{2+}]_i$ is 5 mM, and $1.3 \pm 0.1 \times 10^{-7} M$ if $[Mg^{2+}]_i$ is 10 mM. For 10 of the oocytes, $\log k$ was less than the value for Ca^{2+} -independent light emission with 5 mM Mg^{2+} ($\log k = -6.25$), which suggests that, for these oocytes at least, $[Mg^{2+}]_i$ was greater than 5 mM. If $[Mg^{2+}]_i$ can vary significantly between

oocytes, then the resting $[Ca^{2+}]_i$ could be substantially lower than $1 \times 10^{-7} M$.

Brief treatment (5–10 min) of mouse oocytes with benzyl alcohol (a local anaesthetic) or ethanol induces activation; that is, meiosis II proceeds and pronuclei form, and over 70% of the activated oocytes can develop into blastocysts when cultured for a further 4 days⁸. On exposure of aequorin-injected oocytes to medium containing an activating concentration of benzyl alcohol (0.3–0.5%) or ethanol (5.0–8.6%), $[Ca^{2+}]_i$ rose to $5 \times 10^{-6} M$ (Fig. 1). A slightly lower concentration of benzyl alcohol (0.2%) did not induce this dramatic change in $[Ca^{2+}]_i$, although a small increase in signal was sometimes detected. Similarly, large

increases in $[Ca^{2+}]_i$ were seen, after delays of up to 1 h, in oocytes held in Ca-free medium (2/3 oocytes), Ca-free medium with 1 mM Sr^{2+} added (2/3 oocytes, Fig. 3C) and normal Ca-containing medium without any experimental intervention (11/54 oocytes). The observation of the Ca^{2+} change in oocytes in Ca-free medium explains the apparent paradox that both the injection of Ca^{2+} (ref. 3) and exposure to Ca-free medium^{9,10} activate oocytes. Ca^{2+} rose to 5×10^{-6} M in oocytes in Ca-free medium with the same time course as spontaneously activating oocytes in medium containing 1.71 mM Ca, suggesting that the increase in $[Ca^{2+}]_i$ is due to the release of Ca^{2+} from internal stores. Sr^{2+} enhances activation in Ca-free medium¹⁰, but does not alter the form of the Ca^{2+} release.

To compare the $[Ca^{2+}]_i$ responses during artificial activation with the response during fertilization, single zona-free oocytes containing aequorin were placed with sperm under the photomultiplier. In three oocytes, a series of 8 to 12 Ca^{2+} transients was observed after delays of between 5 and 90 min (Fig. 2). These transients occurred over a period of 45 min in two of the oocytes and 90 min in the third, and preceded a large $[Ca^{2+}]_i$ rise similar to that seen during artificial activation (Fig. 3A, B). The first two or three transients had 4–6 oscillations of large amplitude. The rapid rise and fall of the transients and the oscillations within each transient are in contrast to the slow rise during artificial activation, and the base level between transients starts to rise towards the end of the series in the first phase of the 'activating' rise in $[Ca^{2+}]_i$ (data not shown). This suggests that the signal during the transients does not arise from the entire oocyte, but rather from a localized region, which may be at the site of sperm-egg fusion or near to the plasma membrane of the oocyte. In the medaka fish oocyte (diameter ~ 1 mm) the $[Ca^{2+}]_i$ increase starts at the site of sperm-egg fusion and then spreads around the cell¹. Each series of transients forms a coherent pattern. Thus we believe that a single sperm can initiate a series of transients; these were never seen when oocytes were

activated in the absence of sperm (35 oocytes). In addition, a single fertilizing sperm induces recurring hyperpolarizations in hamster oocytes¹¹, which could be caused by cyclic increases in $[Ca^{2+}]_i$ corresponding to the Ca^{2+} transients reported here. It is therefore likely that the series of Ca^{2+} transients were induced by fertilizing sperm, although it was not possible to confirm that sperm had penetrated the oocytes.

The rise in $[Ca^{2+}]_i$ during activation is piece-wise exponential; that is, pCa ($-\log [Ca^{2+}]_i$) increases linearly with time within successive phases (Figs 1a, b, and 3). In some cases four phases were discernible, giving the appearance of a zigzag (Figs 1, 3C). In one case (see Fig. 3B) there was just one phase. Linearity is seen, to within observational accuracy, from $pCa = 6.4$ to 5.4, that is, during a 10-fold increase in $[Ca^{2+}]_i$. Linearity of $\log k$ within phases was consistently observed. The simplest interpretation is that $[Ca^{2+}]_i$ rose uniformly throughout the whole cytoplasm, in which case the rise within each phase was exponential, and therefore the rate of Ca^{2+} increase was proportional to $[Ca^{2+}]_i$. If $[Ca^{2+}]_i$ is not uniform, the interpretation of k becomes complex, particularly as k is a nonlinear function of $[Ca^{2+}]_i$, and a model to account for linearity in $\log k$ would have to include the diffusion of aequorin as well as Ca^{2+} . While the light signal reached a peak and then dropped rapidly $\log k$ was often still rising linearly (for example, as in Fig. 1a). The fall in light signal was due to the consumption of aequorin. In this circumstance the dependence of the rate of Ca^{2+} increase on $[Ca^{2+}]_i$ necessary to account for the linearity in $\log k$ would become highly contrived if it were assumed that $[Ca^{2+}]_i$ was significantly non-uniform. Step changes in the slope of the graph of pCa could be generated by step changes in the Ca^{2+} release parameter (the ratio of the net rate of Ca^{2+} release to $[Ca^{2+}]_i$, denoted by R_{Ca}). A biochemical system such as that proposed for biochemical oscillators¹² might determine the value of R_{Ca} . Alternation between stable states of this system might cause step changes in R_{Ca} . A step increase in R_{Ca} which raises the rate of

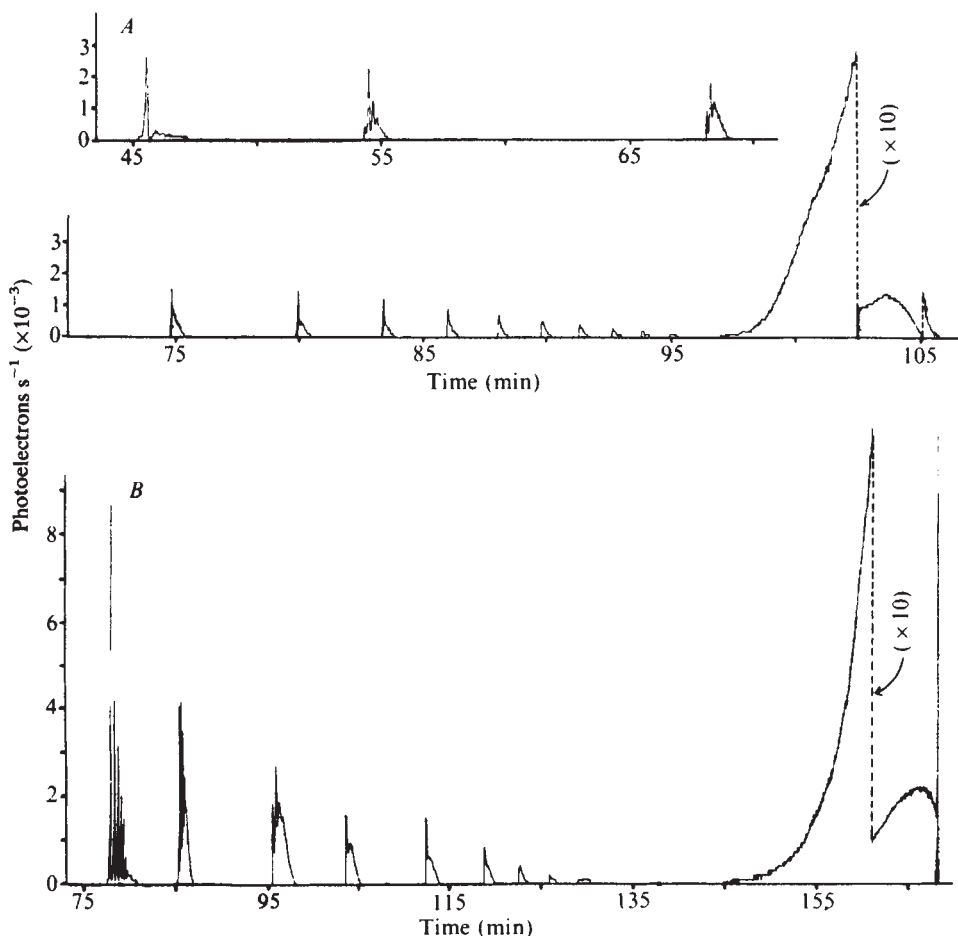


Fig. 2 Aequorin signals (photoelectrons s^{-1} against time) from single zona-free oocytes in the presence of sperm ($\sim 10^6$ per ml), recorded with digital photon-counting equipment. **A**, Sperm were added at 0 min. The first Ca^{2+} transient is seen at 45 min, and a series of transients then follows before the dramatic Ca^{2+} rise beginning at 97 min. Recorder sensitivity was reduced 10-fold at 102 min and returned to normal at 105 min. The trace ends at 106 min, the aequorin having been consumed completely. **B**, A similar experiment recorded at a slower chart speed. The trace has been re-drawn from 85 to 135 min to give a constant time scale. Note the oscillations in the first two transients. (The constant lower limit of the oscillations in the first transient is an artefact of the digital recording.) Throughout the series the transients decline in peak height and duration, and occur more frequently later in the series. The last few transients in each series are superimposed on a slow exponentially rising phase in the base level of $[Ca^{2+}]_i$, recorded at high gain from the analog output of the photon counter. This is not seen on the digital recording shown.

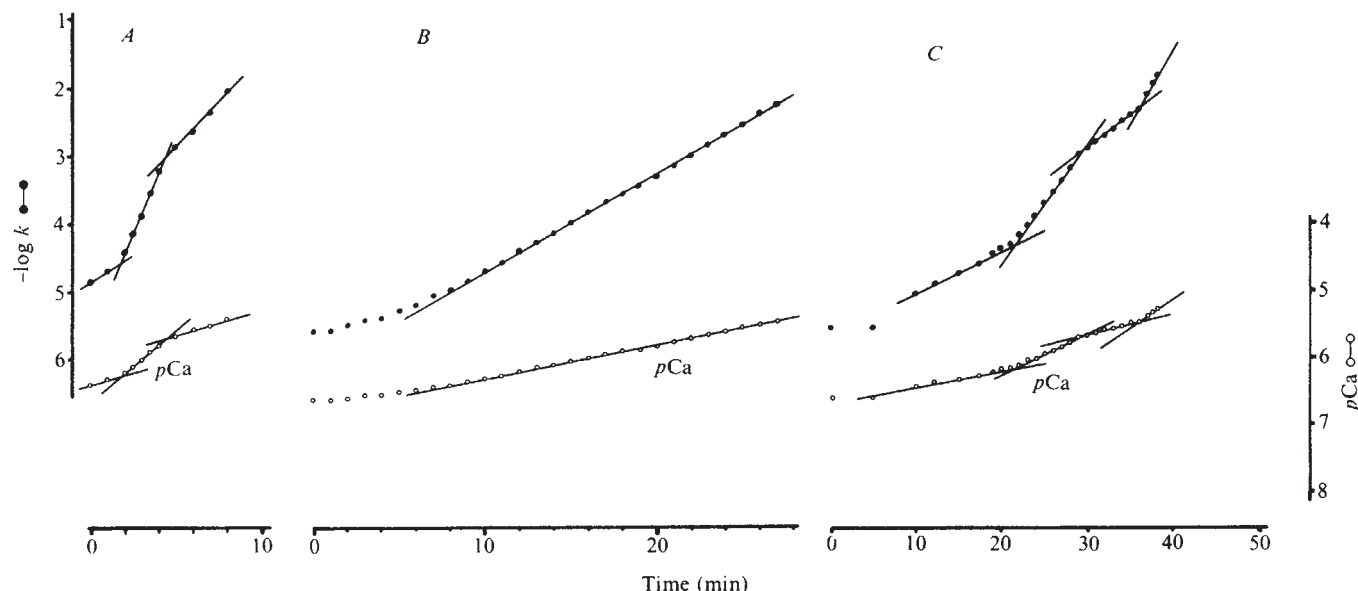


Fig. 3 A, B, fertilization data from Fig. 2 transformed into plots of $-\log k$ (●) and pCa (○) against time (see Fig. 1 legend for details), for the period of the large, 'activating' rise in $[Ca^{2+}]_i$. C, plots of $-\log k$ (●) and pCa (○) against time for the $[Ca^{2+}]_i$ rise in an oocyte in Ca-free medium 16 containing 1 mM $SrCl_2$. At the start of the plots shown, the oocyte had been in this medium for 45 min, with no change in the signal. Straight lines have been drawn through the points to show the piece-wise linearity of the plots.

Ca^{2+} release above that of Ca^{2+} sequestration would initiate an exponential rise in $[Ca^{2+}]_i$. This might be the first event in the process of activation, rather than a rise in $[Ca^{2+}]_i$ above a threshold. The probability of this event occurring may be increased by changes in $[Ca^{2+}]_i$ near the plasma membrane. This local Ca^{2+} concentration may fall in Ca-free medium (because of reduced Ca^{2+} influx) and rise with benzyl alcohol treatment and the Ca^{2+} transients seen in the fertilization experiments. Thus the initial effects of activating stimuli are various, but all serve to destabilize the system controlling R_{Ca} . Therefore the rises in $[Ca^{2+}]_i$ that result are similar.

Here we have given direct evidence that a large increase in $[Ca^{2+}]_i$ is an early event during activation of mouse oocytes. The time course of the $[Ca^{2+}]_i$ increase is an order of magnitude slower than the similar $[Ca^{2+}]_i$ increase seen in medaka¹ and sea urchin² oocytes. The data suggest that this $[Ca^{2+}]_i$ change depends on positive feedback which is itself controlled by an underlying system. An understanding of this system will be needed to explain fully the phenomenon of activation by various stimuli¹³. The technique used here to measure $[Ca^{2+}]_i$ in single mouse oocytes might provide a suitable model system for other cells where changes in $[Ca^{2+}]_i$ are important.

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β -Endorphin and dynorphin control serum luteinizing hormone level in immature female rats

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The suppressive effect of opioid administration on secretion of luteinizing hormone (LH) probably originates primarily in the hypothalamus^{1–5}. Furthermore, the endogenous ligands of the opiate receptors (endorphins) seem to exert a tonic inhibition on the hypothalamic-pituitary-LH axis, as blockade of opiate receptors by the narcotic antagonist naloxone causes a pronounced increase in the release of LH^{3,4}. However, it is unknown which of the particular endorphins present in the hypothalamus is responsible for this tonic inhibition. In addressing this issue we hypothesized that a similar effect to that obtained by blocking receptors with naloxone would be achieved by reducing the concentration of a specific endorphin at the receptors using antibodies against specific opioid peptides. We report here that both anti- β -endorphin antibodies and anti-dynorphin_{1–13} antibodies, when injected into the arcuate nucleus of the mediobasal hypothalamus (MBH) of immature female rats, caused an increase in serum LH. Anti-methionine-enkephalin (Met-enkephalin) antibodies did not affect LH levels. We conclude that β -endorphin and dynorphin rather than Met-enkephalin have a role in regulation of LH secretion in immature female rats. These may represent the first data ascribing a specific physiological function to a particular endorphin.

Morphine, the enkephalins and β -endorphin exert their actions in the MBH by modulating the LH secreting activity of the pituitary^{3,5}. The tonic inhibition effected by the endorphins

has been most convincingly documented in immature female rats: a dramatic (10-fold) increase in serum LH levels is seen within a few minutes of naloxone administration^{6,7}. Assuming a competitive antagonism between naloxone and endorphins, the question remains as to which of the endogenous ligands of the opiate receptors actually controls the hypothalamus-pituitary-LH axis. This is of particular interest in view of the existence of a number of endorphins in the MBH⁸⁻¹¹, which most probably correspond to multiple opiate receptors. In this respect, naloxone cannot provide more precise information as it acts almost equally well on each of the different opiate receptors. A promising system in which to investigate this issue seems, therefore, to be the extremely naloxone-sensitive hypothalamus-pituitary-LH axis of immature female rats. Microinjections into the MBH of specific anti-endorphin antibodies should neutralize the endorphins to give an elevation of serum LH, thus revealing the type of endorphin responsible for the tonic inhibition. This technique has been used successfully in the elucidation of different physiological mechanisms by administering antibodies either centrally¹²⁻¹⁴ or peripherally¹⁵⁻¹⁸.

Anti- β -endorphin (titre 1:50,000; 50% binding of 15 fmol ¹²⁵I tracer), anti-dynorphin₁₋₁₃ (1:20,000) and anti-Met-enkephalin (1:1,000) antisera were obtained and partially characterized as described elsewhere^{10,19,20}. As the titres of anti-dynorphin and anti-Met-enkephalin antisera were relatively low, we isolated the respective immunoglobulins by means of Protein A-Sepharose²¹ and concentrated them to a final titre of 1:80,000. Anti- β -endorphin antiserum (1:50,000) at a concentration of 1 μ l was able to bind ~6 pmol ¹²⁵I- β -endorphin. The corresponding values were 8 pmol for the concentrated anti-dynorphin antibody (1:80,000) and 10 pmol for anti-Met-enkephalin antibody (1:80,000). The affinities of the antisera for their respective antigens were anti- β -endorphin, 7.0×10^{11} l mol⁻¹; anti-dynorphin 1.1×10^{10} l mol⁻¹; and anti-Met-enkephalin, 2.1×10^{10} l mol⁻¹. CN-activated Sepharose 4B coupled to β -endorphin, dynorphin₁₋₁₃ and Met-enkephalin, respectively, was used to remove selectively the antibodies from the anti- β -endorphin antiserum or the solutions containing the concentrated anti-dynorphin or anti-Met-enkephalin antibodies¹⁹. The remaining solutions completely lost their binding capacity and served as controls.

The powerful effect of naloxone (2.5 mg per kg s.c. (subcutaneous)) on serum LH levels in naive immature female rats^{6,7} is confirmed here. LH levels increased to 420 ± 21 ng ml⁻¹ ($n = 16$) in 12-day rats (saline controls: 43 ± 19 , $n = 24$) 30 min after naloxone treatment and to 381 ± 16 ng ml⁻¹ ($n = 11$) in 14-day rats (controls: 47 ± 15 , $n = 16$). In 12-day female rats anaesthetized with hexobarbital (100 mg per kg s.c.) the antagonist increased the LH level to 370 ± 25 ng ml⁻¹ ($n = 39$) (saline controls: 62 ± 21 , $n = 27$).

In further experiments, unilateral microinjections (1 μ l) of specific anti-endorphin antibodies were performed only on 12-day female rats anaesthetized with hexobarbital (100 mg per kg s.c.). After 40 min the rats were decapitated, the blood collected and the brains removed. The serum LH concentrations determined were compared with the site of injection in the MBH, identified by histological examination. Figure 1 shows LH levels in rats after microinjection of different antibodies into distinct areas of the MBH. Figure 1a shows results for anti- β -endorphin antiserum (titre 1:50,000); of nine areas injected, only injections into the arcuate nucleus were followed by an increase in LH (fourfold). A similar pattern was observed for anti-dynorphin₁₋₁₃ antibody (titre 1:80,000; Fig. 1b). Although the LH response after injection into the arcuate nucleus was less (twofold) than that observed with anti- β -endorphin antiserum, the effect was highly significant ($P < 0.01$). Furthermore, LH levels doubled ($P < 0.01$) on injection of anti-dynorphin antibody into the nucleus dorsomedialis (pars ventralis); the control values for this area were the lowest of all obtained. Thus, although there was a statistically significant increase in LH in this case, the functional relevance of these data is questionable. In general, the control values were greater than those shown in

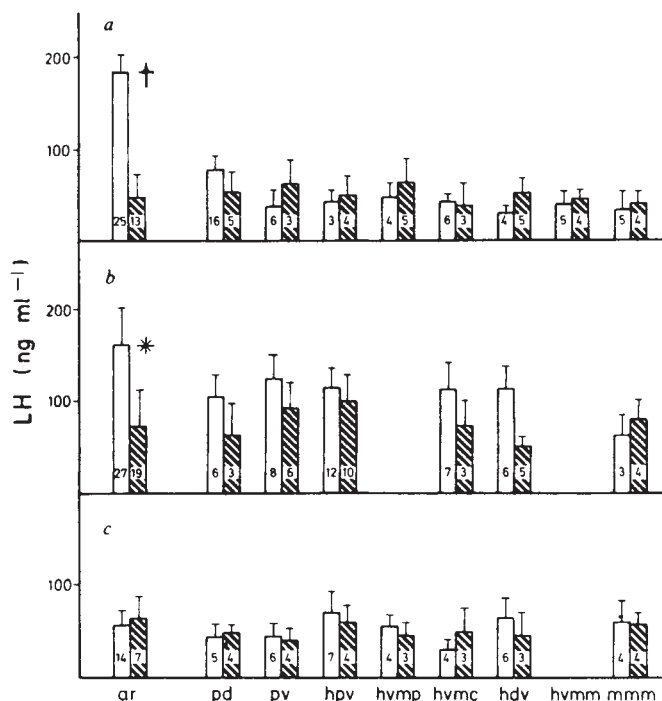


Fig. 1 Serum LH levels in 12-day-old female rats. The anaesthetized animals (hexobarbital, 100 mg per kg s.c.) were microinjected with: *a*, 1 μ l anti- β -endorphin antiserum (titre 1:50,000); *b*, anti-dynorphin₁₋₁₃ immunoglobulin-containing solution (1:80,000); or *c*, or anti-Met-enkephalin immunoglobulin-containing solution into different areas of the mediobasal hypothalamus (open columns). The hatched columns represent data from rats injected with 'carrier' solutions lacking the specific endorphin immunoglobulins. The animals were decapitated 40 min after the stereotactically guided administration of the antibodies (5 μ l Hamilton syringe, flat tip of the needle). Pilot studies on rats killed 20 min after antibody injection did not reveal changes in serum LH levels. The antibodies were injected within 3 min and the needle left in place for a further 3 min; experiments were done between 9.00 and 13.00 h. The trunk blood of the decapitated animals was collected, and the serum collected after 30 min at room temperature by centrifugation. The samples were stored at -20°C and assayed in duplicate for LH as described in detail elsewhere²⁶, using reagents provided by the Pituitary Agency of the National Institutes of Health. Variability within assays was 10% at an average level of 41 ng LHRP-1 (luteinizing hormone rat pituitary standard) per ml. The cannula position was verified by histological examination according to König and Klippel²⁷. Apparently, driving the needle through the median eminence caused extremely high serum LH levels (>800 ng ml⁻¹). Anti- β -endorphin antibodies labelled with ¹²⁵I by the Chloramine-T method were used to study their diffusion within the hypothalamus. Punching the tissue around the site of injection revealed that the antibodies penetrated less than 1.8 mm from the site of injection within 40 min. The columns represent mean serum LH levels of different animals (number of animals shown within columns). Vertical bars indicate s.e. Significance of differences was calculated according to the two-tailed Student's *t*-test (* $P < 0.01$; † $P < 0.001$). Data are given only for more than two injections into a certain area. The areas injected are indicated on the abscissa, abbreviated according to König and Klippel²⁷: ar, nucleus arcuatus; pd, nucleus preamillaris dorsalis; pv, nucleus preamillaris ventralis; hpv, nucleus periventricularis (hypothalamus); hvmp, nucleus ventromedialis (hypothalamus), pars posterior; hvmc, nucleus ventromedialis (hypothalamus), pars centralis; hdv, nucleus dorsomedialis (hypothalamus), pars ventralis; hvmm, nucleus ventromedialis (hypothalamus), pars medialis; mmm, nucleus mammillaris medialis, pars medialis.

Fig. 1a, c. We assume that this reflects the presence of impurities due to the processing of the anti-dynorphin antiserum. In contrast to the data obtained with anti- β -endorphin and anti-dynorphin antisera, none of the areas injected with anti-Met-enkephalin antibody (titre 1:80,000) responded with an increase in serum LH concentration (Fig. 1c). These results were obtained for high concentrations of antibodies (anti- β -endorphin titre 1:50,000; anti-dynorphin₁₋₁₃ and anti-Met-enkephalin, each 1:80,000). Injections of 1 μ l anti- β -endorphin antiserum diluted 10-fold (1:5,000) did not raise LH concentrations. Similarly, anti-dynorphin₁₋₁₃ antiserum (titre

1:20,000; not enriched) or anti-Met-enkephalin antiserum (1:1,000) failed to increase LH levels.

The observations reported here support the concept of a tonic inhibitory effect of endorphins on the hypothalamus–pituitary–LH axis. Two findings are of particular importance. First, LH levels are affected not only by anti- β -endorphin antibodies but also, although to a lesser degree, by anti-dynorphin₁₋₁₃ antibodies. Second, the arcuate nucleus was the only sensitive region in a number of areas of the MBH tested. The fact that dopamine-containing neurones and β -endorphin-containing neurones are closely associated in the arcuate nucleus²², together with our present findings, supports the notion of an interaction between endorphinergic and dopaminergic mechanisms in the modulation of the release of LH-releasing hormone from the median eminence^{1,2,5}. The experiments implicating dynorphin in the control of serum LH via the arcuate nucleus are less compelling than the data suggesting a role for β -endorphin. However, in support of a regulatory role of dynorphin₁₋₁₃ are its high immunoreactive content in this area (1–5 pmol per mg protein)⁹⁻¹¹ and its extremely high potency²³.

The finding that Met-enkephalin probably does not regulate serum LH in immature female rats is of interest in view of the conflicting data obtained from adult rats. Goldberg *et al.*²⁴ reported that Met-enkephalin had no effect on serum LH concentrations, whereas others^{1,2} have observed an inhibitory action of Met-enkephalin on the release of LH-releasing hormone in mediobasal hypothalamic slices. However, the Met-enkephalin concentrations used in these studies were far greater than the K_d value for Met-enkephalin (0.66 nM)²⁵, therefore other receptors could also be activated by this opioid peptide. The general question remains whether mechanisms responsible for modulation of LH release in adult rats are similar or identical to those which operate in 12-day-old female rats.

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Effects of nifedipine on electrical and mechanical responses of rat and guinea pig vas deferens

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The mechanical response of the vas deferens to single pulses or short trains of stimuli can be resolved into two distinct phases, an initial twitch response (*I*) attributed to the release of an unknown transmitter and a slower better maintained secondary contraction (*II*)^{1,2}, attributed to noradrenaline release. Recently, it has been shown that the calcium antagonist nifedipine³ eliminates the initial twitch response leaving the secondary component intact^{4,5} and we have now examined the electrical responses underlying these mechanical events. We show here that nifedipine abolishes the smooth muscle action potential and the initial twitch response (*I*) without reducing the excitatory junction potential (e.j.p.), suggesting a fundamentally different basis of the two responses and illustrating the pharmacological value of nifedipine.

In rat and guinea pig, the contractile response of the vas deferens to a single field stimulus to the sympathetic innervation consisted of several components. In the rat this response could be separated into two components; a rapid, early (200–300 ms) response (*I*_s) and a slower (600–700 ms) response (*II*_s). The α_1 -adrenoreceptor antagonists, prazosin (10^{-7} M) or corynanthine (3×10^{-6} M), blocked *II*_s but left *I*_s intact. In contrast, nifedipine (10^{-5} M) together with α_1 -adrenoreceptor antagonists virtually eliminated the whole response (Fig. 1).

In the guinea pig, where separation of the mechanical response to single stimuli by time course into two distinct components is less clear than in the rat^{6,7}, corynanthine had little effect but nifedipine reduced the response by 40–70% and, by producing a relatively greater inhibition of the early part of the contraction, altered the time course. Thus, in each species the contractile response to a single stimulus contained a nifedipine-sensitive but α_1 -antagonist-resistant component (*I*_s) and an α -noradrenergic component (*II*_s).

In each species, a train of 25 pulses at 6 Hz produced a biphasic concentration, a rapid, early (1–2 s) component (*I*_t) followed by a second component (*II*_t), which lasted until stimulation ceased, *I*_t and *II*_t being relatively dominant in the prostatic and epididymal portions, respectively^{6,7}. In each species, corynanthine (3×10^{-6} M) produced inhibition which was greater in *II*_t but which, again, did not exceed 50%. In contrast, nifedipine, although reducing each component, produced a particularly marked reduction of up to 90% in *I*_t. The effects of nifedipine and corynanthine were additive but did not result in complete abolition of the response. Thus, in each species, each phase of the response contained a nifedipine-sensitive component which was a dominant factor in *I*_t. In contrast the component attributed to α -noradrenergic activation contributed mainly to *II*_t, particularly in the guinea pig where the noradrenergic component to a single pulse is less obvious. The nifedipine-sensitive component corresponded in drug sensitivity, time course and species specificity to the nerve-induced 'non-noradrenergic' contraction^{1,2,6,7}.

In separate experiments on the guinea pig vas deferens, e.j.p.s and action potentials were recorded using intracellular micro-electrodes (20–40 M Ω filled with 3M KCl) following pre- and

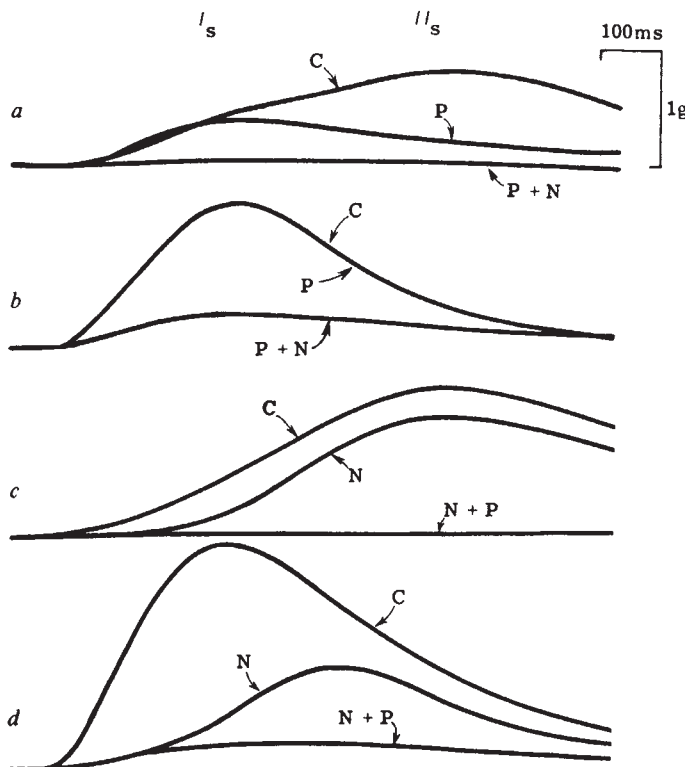


Fig. 1 Effects of drugs on the time courses of the isometric contractions of isolated portions of rat vas deferens to single supramaximal field stimuli (0.5 ms). The trace was triggered by the stimulus. *a*, The epididymal and *b*, the prostatic portions of a vas deferens. Control (C) was followed by responses in the presence of prazosin (10^{-7} M) (P) and then with the further addition of nifedipine (10^{-5} M) (P + N). In this particular experiment, prazosin had no effect at all in *b* so C and P coincide. *c*, *d*, The contralateral vas—as for *a* and *b* except that nifedipine (N) was administered first and was followed by the addition of prazosin (N + P). I_s and II_s indicate the times of the peaks of the two phases of contraction.

postganglionic hypogastric nerve stimulation with trains of stimuli at 5–10 Hz, pulse width 0.2 ms and supramaximal voltage⁸.

In all preparations, trains of stimuli at 5 Hz evoked e.j.p.s which by facilitation and summation reached threshold for the initiation of a muscle action potential and the vas deferens contracted violently, normally dislodging the microelectrode. The muscle action potential had an overshoot of >15 mV in all control cells impaled. At 10–15 min after nifedipine (10^{-5} M to 3×10^{-5} M), the e.j.p. was not reduced, and if anything was enhanced. Trains of stimuli now produced a depolarization which greatly exceeded the control threshold, but it was not possible to induce muscle action potentials (Fig. 2) even with trains of stimuli at 10 Hz. Nifedipine reduced the rate of rise and fall of the spike before complete block occurred (Fig. 3). During long trains of stimuli at 5 and 10 Hz, the microelectrode was dislodged from the cell presumably due to contraction of the vas deferens, even though no action potentials were recorded. After wash-out (15 min) the muscle action potential began to return (Fig. 2) although the rate of rise was still greatly reduced compared with the control.

The α -adrenoreceptor antagonist prazosin (10^{-6} M) did not reduce the muscle action potential but increased e.j.p. amplitude. Prazosin did not prevent the effects of nifedipine (3×10^{-5} M), suggesting that nifedipine is not acting via an α -adrenoreceptor.

Previously, it has not been possible to block selectively the initial twitch and leave the secondary response (II) intact. We have now shown that nifedipine blocks the initial twitch (I) and prevents the initiation of the smooth muscle action potential.

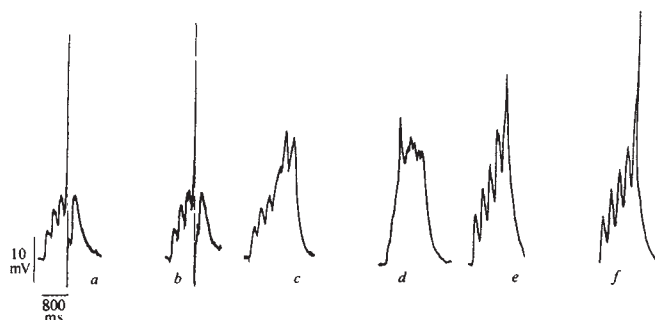


Fig. 2 Effects of nifedipine (10^{-5} M) on excitatory junction potentials and action potentials evoked in the guinea pig vas deferens by hypogastric nerve stimulation (pulse width 0.2 ms, supramaximal voltage). Stimulus artefacts have been removed from the trace. *a*, *b*, Controls, resting membrane potential (RP) –57 mV and –56 mV respectively. Response evoked by trains of five pulses at 5 Hz. *c*, 15 min after nifedipine; RP = –62 mV, six pulses at 5 Hz. *d*, Same cell as *c*, 10 pulses at 10 Hz. *e*, Wash 10 min; RP = –63 mV, five pulses at 5 Hz. *f*, Same cell as *e*, 15 min wash; five pulses at 5 Hz. Records *a*/*b*, *c*/*d* and *e*/*f* are taken from three neighbouring cells in the same preparation.

Nifedipine is known to be a calcium antagonist without local anaesthetic activity⁹ and it is also known that calcium is the ion carrying most of the inward current during the rising phase of the action potential^{10–12}. The initial twitch response (I) therefore corresponds to the contraction associated with the smooth muscle action potential and associated contraction, and is resistant to concentrations of α -adrenoreceptor antagonists which block the noradrenergic contraction, it is probable that the α -adrenoreceptor mediated contractile response of the vas deferens does not require an action potential.

These results show there to be a fundamental difference between the two types of response to nerve stimulation in the vas deferens. If noradrenaline is the sole transmitter then it

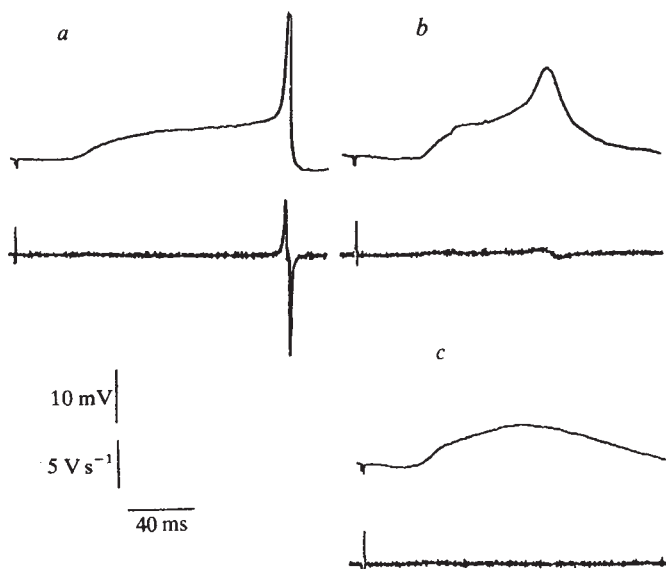


Fig. 3 The effects of nifedipine (10^{-5} M) on action potentials in the guinea pig vas deferens. Action potentials were evoked by stimulation of the hypogastric nerve with trains of five stimuli at 5 Hz. The records show the membrane response initiated by the fourth stimulus in a train. The upper record is the d.c. response calibration bar 10 mV; lower record, the differential of the d.c. signal, calibration bar 5 V s^{-1} . *a*, Control, resting membrane potential (RP) –62 mV; *b*, 10 min after nifedipine, RP = –61 mV; *c*, 20 min after nifedipine, RP = –64 mV. Records *a*, *b* and *c* are taken from three neighbouring cells in the same preparation.

produces its contractile effects through two processes; the first involves e.j.ps which summate to initiate propagated action potentials and does not involve α -adrenoreceptors; the second does not involve e.j.ps or propagated action potentials but is mediated by α_1 -adrenoreceptors. The only experimental obstacle which remains to acceptance of noradrenaline as sole transmitter is the survival of the first process after reserpine or chemical sympathectomy¹³. The results provide further proof that the different types of response in the two ends of the vas result from differences in the dominant transmission process.

These results in vas deferens show some similarities with recent data derived from arteriolar smooth muscle^{14,15}. First, in

each case the e.j.p. was found to be resistant to α_1 -adrenoreceptor antagonists. Second, in the responses which are mediated by α_1 -adrenoreceptors in these tissues, there is a similarity between the contractile response of the arterioles to iontophoretically applied noradrenaline, which occurred without any detectable change in membrane potential, and the 'adrenergic' response to nerve stimulation in the vas, which occurred independently of the action potential. This suggests that in smooth muscle there is a type of α_1 -adrenoreceptor which may directly activate the contractile process without utilising the nifedipine-sensitive influx of calcium associated with the rising phase of the action potential.

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Vertical organization of neurones accumulating ³H-GABA in visual cortex of rhesus monkey

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Electrophysiological and pharmacological studies^{1–6} indicate that the specific responses of most visual cortical neurones depend on intracortical γ -aminobutyric acid (GABA)-mediated inhibitory processes. GABAergic interneurons have been visualized in all layers of the mammalian cerebral cortex by immunocytochemical methods^{7,8} and by high-affinity uptake of exogenous ³H-GABA^{9–11}. It is recognized that GABA is synthesized and specifically accumulated by aspiny and sparsely spinous stellate cells, but there is no evidence available to indicate whether the laminar distribution of these cells and their axonal projections are related to the known role of GABAergic inhibitory processes in the generation of responses in visual cortical cells. It would therefore be of value to delineate the intracortical projection of the axons of different types of GABA-releasing neurones in regions of cortex where the receptive field properties of the neurones, and their modification by GABA antagonists, are well known. The selective high-affinity uptake of labelled GABA has been useful in delineating GABAergic systems¹²; recently, it has been shown that exogenous ³H-GABA is specifically taken up and transported retrogradely by axons of neurones thought to be GABAergic^{13,14}. Using microinjections of ³H-GABA into different layers of the monkey visual cortex, we have examined the pattern of labelled neurones. We report here a bimodal distribution of GABA-accumulating neurones after injection into layers V and VI, with one group of neurones around the injection site and the other directly above, in layers II and III. We provide evidence that the latter neurones are non-pyramidal cells, probably labelled by retrograde axonal transport from the deep layers.

Two adolescent male macaque monkeys were used. One had taken part in behavioural tests of memory and had received surgical section of the fornix 6 months before the present study. The other had a high antibody titre to *Herpes simiae* and could therefore not be used in long-term experiments. There was no reason to suppose that the visual cortex of either monkey was

abnormal. The animal was sedated with an intramuscular injection of ketamine hydrochloride (10 mg per kg; Ketalar, Parke-Davis) and then deeply anaesthetized with an intravenous injection of sodium pentobarbitone (Sagatal, May and Baker). After exposing the lateral surface of one occipital lobe, the cortex was injected with ³H-GABA (0.33 mM, 60 Ci mmol⁻¹; Radiochemical Centre) dissolved in Krebs bicarbonate¹⁵. All the injections were delivered using glass micropipettes (30–50 μ m tip diameter) penetrating at a very oblique angle to the surface of the cortex. The pipette was advanced 7–9 mm from the pia and 0.1 μ l (2 μ Ci) ³H-GABA injected by pressure¹⁶ at each of four to six sites at 1–2 mm intervals along the injection track as the capillary was gradually withdrawn. In the first monkey the GABA injection track (no. 1) in area 17 passed obliquely through layers V and VI and ended in the white matter. In the other monkey one injection track (no. 2) in area 17 advanced obliquely from layer I to layer IVA, and another injection track (no. 3) in area 18 (posterior lip of the lunule sulcus) included all layers and the white matter as the capillary proceeded gradually deeper. After a post-injection survival time (no. 1, 40 min; no. 2, 55 min; no. 3, 35 min) the animals were perfused with fixative and slices of the injected cortex processed for Golgi staining and gold toning, as described previously¹⁶. Semi-thin (1 μ m) sections perpendicular to the injection track were cut from the Golgi sections and processed for autoradiography¹¹. Golgi staining was used to reveal the processes of some of the GABA-accumulating cells, but because of the unreliability of the staining, no unequivocal identification of the cell type of neurones labelled in the upper layers has yet been obtained.

Neurones in each of layers I–VI in both areas 17 and 18 became labelled when the layer was injected with ³H-GABA. The number of labelled cells gradually decreased in all directions from the injection track, but labelled cells could always be found above the capillary track up to and including layer II. A distinct, bimodal pattern of labelled neurones and fibres was observed when the injection track passed through lower layer V, layer VI and the superficial white matter (injections 1 and 3; Fig. 1a). Large labelled neurones were seen around the injection site with scattered neurones as far as 1–1.5 mm from the injection site in layers V and VI. Occasionally, labelled neurones were found in layer IV and lower layer III, always above the injection track. In addition, another group of strongly labelled small, mainly fusiform cells was observed in layers II and upper III (Fig. 2a, b). The quantitative distribution of the neurones labelled by one oblique penetration in area 17 is shown in Fig. 1b.

It is unlikely that the neurones in the upper group were labelled by GABA taken up from the extracellular space in the upper layers, because there were very few labelled neurones in lower layer III and layer IV, although these layers contain a

large number of GABA-accumulating neurones when injected directly. A more likely explanation is that the neurones in layers II and upper III were labelled by retrograde axonal transport of ^3H -GABA from the injection site in the deep layers. This is supported by the observation that strongly labelled fibre bundles pass through layers IV and lower III (Fig. 2c).

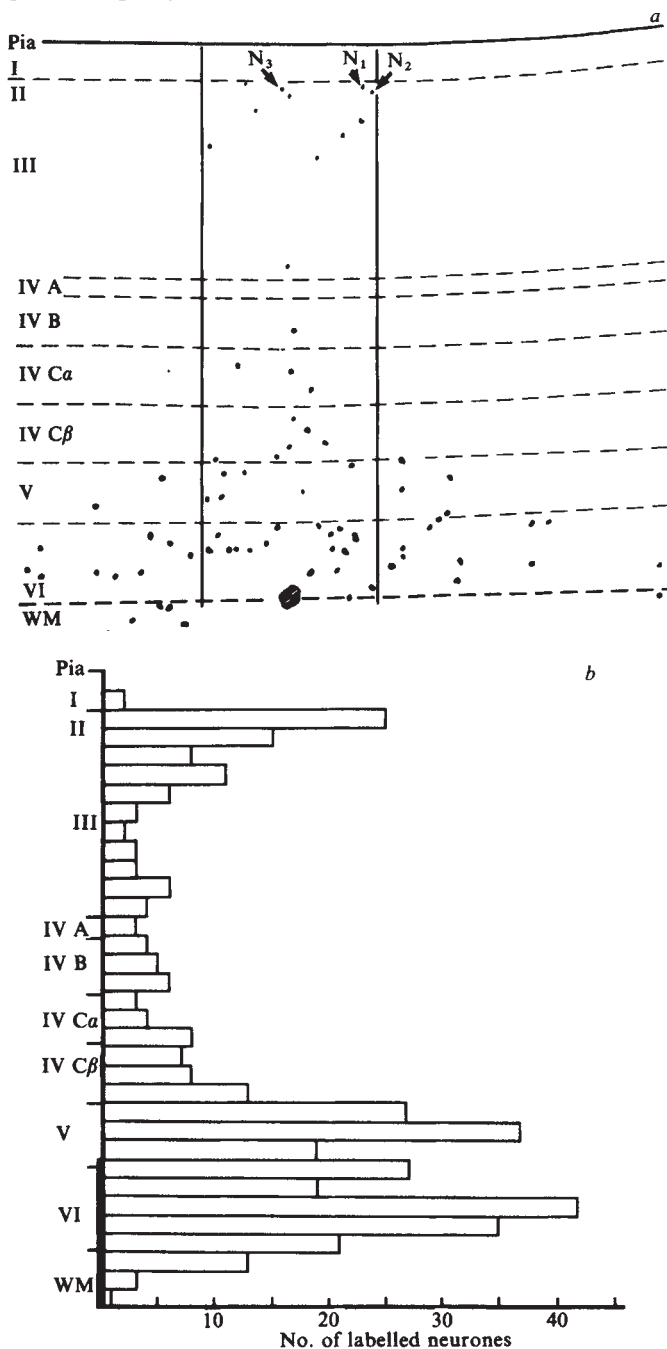


Fig. 1 *a*, Location of labelled neuronal perikarya (dots) in a single section of monkey striate cortex after ^3H -GABA injection at the border of layer VI and the white matter. The drawing is of a section perpendicular to the pial surface; the injection was made obliquely at an angle of almost 90° to the plane of the section. Besides the large labelled neurones around the injection track (shaded area) and a few neurones in layer IV, there is a group of small labelled neurones in layer II and upper layer III. N_1 - N_3 are shown on light micrographs in Fig. 2. Note the vertical alignment of labelled neurones above the injection track. The vertical lines enclose a $500\ \mu\text{m}$ column in which cells were counted for *b*. *b*, Distribution of 393 ^3H -GABA-labelled cells in all layers (I-VI) of the striate cortex, counted in 13 semi-thin sections cut perpendicular to the injection track at different levels of injection no. 1. Thick vertical line represents the levels where ^3H -GABA was injected. WM, white matter.

What type of neurones in the upper layers accumulate GABA from the deeper layers? There is a very strong projection from layer II to layer V in area 17 of the monkey, mediated by small pyramidal cells¹⁷. Thus the terminals of these neurones could take up the injected GABA. As pyramidal and stellate neurones can be differentiated on the basis of fine structural criteria, we studied the characteristics of six labelled neurones from the upper layers. After localizing the labelled neurones in semi-thin sections, a series of 84 and 132 ultrathin sections, respectively, were cut from two blocks containing the remainder of the perikarya of the labelled neurones. Using electron microscopy we found that labelled neurones had an indented eccentric nucleus with dense clumps of chromatin. There were many free polyribosomes in the cytoplasm, making the cell conspicuously electrodense. The thin dendrites emerging from the perikarya contained densely packed microtubules (Fig. 2d) and some of the dendrites were beaded, receiving predominantly asymmetric

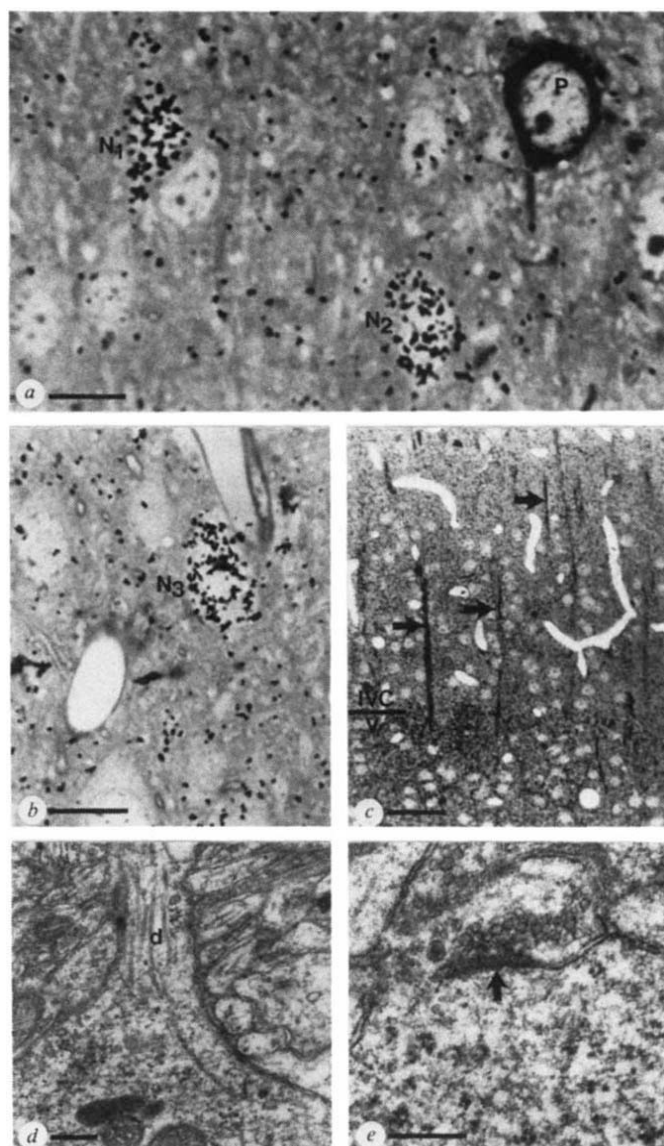


Fig. 2 Light (*a*-*c*) and electron microscopic (*d*, *e*) details from the area shown in Fig. 1a. *a*, *b*, Semi-thin sections of ^3H -GABA-labelled neurones (N_1 - N_3) in layer II, which were also serially sectioned for electron microscopy. One Golgi-stained pyramidal cell (P) is also present. *c*, Vertical labelled fibre bundles (arrows) passing through layer IVC. *d*, Origin of a small dendrite (*d*) from the neurone N_3 directed towards the pia. *e*, Asymmetric synapse on the perikaryon of the labelled neurone N_2 . *a*-*c*, Ilford K5 emulsion; 30 days exposure. Scale bars: *a*, *b*, $10\ \mu\text{m}$; *c*, $50\ \mu\text{m}$; *d*, *e*, $0.2\ \mu\text{m}$.

synapses. The perikarya received few, mainly symmetrical synapses from boutons containing pleomorphic vesicles, but occasional asymmetric synapses established by boutons containing round synaptic vesicles were also observed (Fig. 2e). All these features are characteristic of small stellate cells in the monkey primary sensory cortex^{18,19}. For comparison we also studied two Golgi-stained, gold-toned small pyramidal cells in layer II. These neurones differed from labelled cells in that the nucleus was large, round and relatively transparent and there were few free ribosomes. Thick dendrites tapered gradually from the perikaryon and an apical dendrite was observed. The perikarya and proximal dendrites received only symmetrical synapses, confirming previous findings^{20,21}.

These data taken together provide evidence for a GABA-accumulating stellate neurone system with perikarya situated in layer II and upper layer III, and with axons descending vertically in bundles into layer V. One stellate neurone, the double-bouquet cell with tight vertical axon bundles described in previous studies²²⁻²⁵, corresponds very well to the above characteristics but more detailed information is required before we can conclude definitely that GABA-accumulating neurones of the upper layers are, or include, double-bouquet cells. It was shown recently²⁵ that this neurone makes symmetrical synaptic contacts, similar to those which contain glutamic acid decarboxylase (GAD) in the monkey²⁶, with dendritic shafts and spines. Although the axons of double-bouquet cells do not enter layer VI the fact that an injection here produces labelled neurones in layers II and III is easily explained by diffusion of the GABA across the V-VI boundary.

The presence of a GABAergic vertical neurone system passing throughout layers II to V of the visual cortex and capable of influencing neurones in layer VI with dendrites in layer V is significant in view of the functional, columnar organization of cortical neurones²⁷. The distribution of GAD immunoreactivity in the plane of the cortical surface was recently shown to follow a pattern resembling ocular dominance columns⁷. On the other hand, it has been shown in area 17 that cortical neurones in a radial column are maximally excited by a bar of light or grating of a particular orientation, and that this orientation specificity is temporarily abolished by the GABA antagonist bicuculline²⁻⁶. The columnar system of GABA-accumulating axons described here may have an important role in this specificity, as the axon bundles are highly restricted in lateral extent (20–50 µm in diameter) and could have a powerful effect locally.

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Agonist and antagonist benzodiazepine receptor interaction *in vitro*

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A representative of a novel series of imidazodiazepines, Ro 15-1788, selectively antagonizes all major central actions of benzodiazepines by competitive, high-affinity interactions with benzodiazepine receptors (BR) in the central nervous system (CNS)¹⁻³. Doses of Ro 15-1788 sufficient to antagonize benzodiazepine actions have been shown *per se* to lack pharmacological action in animals and man¹⁻⁴. Thus Ro 15-1788 provides a highly selective tool for experimental investigations of BR-mediated events and is of therapeutic value in all cases where a rapid termination of benzodiazepine actions is indicated. Using ³H-labelled Ro 15-1788 as radioligand in equilibrium binding studies *in vitro*, we show here that ³H-Ro 15-1788 interacts with the same number of BR sites as the agonist ³H-clonazepam in various brain regions. However, their mode of receptor interaction is different. In conditions which alter receptor affinity for ³H-clonazepam binding, such as addition of γ -aminobutyric acid (GABA) or certain ions, no change is seen in ³H-Ro 15-1788 binding. This effect can be used to distinguish between benzodiazepine receptor agonists and antagonists *in vitro*. Furthermore, the thermodynamics of agonist and antagonist receptor interaction are different, but only at temperatures above 21 °C.

Several lines of evidence indicate that ³H-Ro 15-1788 interacts with the same central type of BR sites as benzodiazepine agonists:

(1) ³H-Ro 15-1788 was bound to the same maximal number of specific binding sites ($B_{\max}$) as ³H-clonazepam in frozen and extensively washed synaptic membrane fractions⁵ prepared from various brain regions. ³H-clonazepam was used as agonist because, in contrast to ³H-flunitrazepam (³H-FNZP) and ³H-diazepam, it binds selectively to the central type of BR without interacting with the peripheral type of benzodiazepine binding sites present in brain tissue⁶. Scatchard analysis of ³H-Ro 15-1788 and ³H-clonazepam specific binding revealed only one population of binding sites in each brain area tested, with no major regional difference in the apparent affinity constant (K_D) for either radioligand (cerebral cortex: ³H-Ro 15-1788 (³H-clonazepam) specific binding, $B_{\max} = 1.7(1.6)$ pmol per mg protein, $K_D = 0.9(0.9)$ nmol l⁻¹; cerebellum: $B_{\max} 0.7(0.7)$, $K_D = 0.8(0.7)$; hippocampus: $1.7(1.6)$, $0.9(0.8)$; hypothalamus: $1.2(1.2)$, $0.9(1.0)$; striatum: $0.9(0.8)$, $1.1(1.0)$; midbrain: $0.9(0.7)$, $1.1(0.9)$; medulla pons: $0.6(0.5)$, $0.8(0.8)$). Values of s.e.m. from at least three determinations of K_D or $B_{\max}$ were in the range ± 0.08 – 0.1 . In ³H-Ro 15-1788 binding the antagonist Ro 14-7437 ($1 \mu\text{mol l}^{-1}$), which is the desfluoro analogue of Ro 15-1788, was used as displacer². ³H-clonazepam was displaced by $10 \mu\text{mol l}^{-1}$ diazepam).

(2) Autoradiographically, the density and distribution of ³H-Ro 15-1788 binding sites in sections of various CNS regions were very similar to those seen with ³H-clonazepam.

(3) A variety of receptor agonists and antagonists showed very similar potency in inhibiting either ³H-clonazepam or ³H-Ro 15-1788 binding (Table 1).

(4) The inhibition of ³H-Ro 15-1788 binding by diazepam or flunitrazepam was competitive¹.

(5) In heat inactivation experiments⁷, the amount of specifically bound ³H-Ro 15-1788 was reduced at the same rate and to the same extent as that of ³H-clonazepam (60 °C, 5–60 min). In the presence of $10 \mu\text{mol l}^{-1}$ of GABA, diazepam or

Table 1 Inhibition of ^3H -clonazepam and ^3H -Ro 15-1788 binding by various BR ligands

Ligand	IC ₅₀ (nmol l ⁻¹)	
	^3H -clonazepam binding	^3H -Ro 15-1788 binding
Agonists		
Clonazepam	1.2	1.4
Flunitrazepam	5.1	4.8
Diazepam	13.5	19.5
(+) Ro 11-6896	15.5	14.5
Zopiclone	46	50
CL 218 872	300	340
Chlordiazepoxide	1,300	1,100
Medazepam	1,900	1,900
(-) Ro 11-6893	>10,000	>10,000
Antagonists		
Selective		
Ro 15-1788	2.0	1.3
Ro 14-7437	4.5	3.0
Non-selective		
CGS 8216	0.3	0.2
Ethyl- β -CC	1.6	2.0
Methyl- β -CC	2.3	1.9

Inhibition of ^3H -clonazepam binding (specific activity 16.1 Ci mmol⁻¹, 0.5 nmol l⁻¹; assay: H.M., in preparation) and of ^3H -Ro 15-1788 binding (specific activity 26.8 Ci mmol⁻¹, 0.5 nmol l⁻¹; ref. 2) by various BR ligands at 0°C using freeze-thawed and extensively washed synaptic membranes⁵ of rat cerebral cortex. The concentrations giving half-maximal inhibition (IC₅₀) are mean values of three determinations with s.e.m. values between ± 5 and 20%. (For the structure of enantiomers Ro 11-6896 and Ro 11-6893, of CGS 8216, CL 218 872 and zopiclone, see refs 27, 22, 16 and 15, respectively.) β -CC, β -carboline-3-carboxylate.

Ro 15-1788, the binding sites for the two radioligands were similarly protected from heat inactivation (60°C, 30 min).

(6) Ro 15-1788 did not interact with the peripheral type of benzodiazepine binding site in the brain as it was unable to displace ^3H -Ro 5-4864 when tested in cerebral cortex (IC₅₀ > 10 $\mu\text{mol l}^{-1}$).

Thus, Ro 15-1788 seems to interact with the same number of sites of the central BR type as benzodiazepine agonists. The mode of interaction of Ro 15-1788 with BR is, however, different from that of receptor agonists. BR affinity for ^3H -diazepam or ^3H -FNZP binding is increased in the presence of various chemicals, including 100 $\mu\text{mol l}^{-1}$ GABA^{8,9,28-30}, 500 $\mu\text{mol l}^{-1}$ pentobarbitone^{10,11}, 10 $\mu\text{mol l}^{-1}$ SQ 20 009 (ref. 12), 2 mmol l⁻¹ NiCl₂ (ref. 13), 20 mmol l⁻¹ NH₄I, 200 mmol l⁻¹ NH₄Cl or 100 mmol l⁻¹ NH₄Br (ref. 14), while the BR affinity is decreased in the presence of 50 mmol l⁻¹ (NH₄)₂SO₄ or 200 mmol l⁻¹ ammonium acetate¹⁴. However, in parallel assays, neither the K_D nor the B_{max} value for ^3H -Ro 15-1788 binding was affected by these agents. This lack of susceptibility of ^3H -Ro 15-1788 binding to conformational changes of BR may reflect the inability of Ro 15-1788 to induce a conformational change of the receptor and, thereby, to trigger a biological response.

The different responses of ^3H -agonist and ^3H -Ro 15-1788 binding to alterations in BR affinity can be used to distinguish *in vitro* between agonists and antagonists; the inhibitory potency of a ligand is determined in ^3H -Ro 15-1788 binding in the presence and absence of a compound such as GABA which is known to alter the affinity for agonists. At 35°C the addition of 100 μM GABA increased two- to threefold the inhibitory potency (IC₅₀) of various BR agonists such as flunitrazepam, diazepam, oxazepam, zopiclone¹⁵ and CL 218 872 (ref. 16) in ^3H -Ro 15-1788 binding, while the inhibitory potency of various antagonists remained unaltered, as shown for the selective antagonists Ro 15-1788 and Ro 14-7437 (Fig. 1) and for CGS 8216 and ethyl- β -carboline-3-carboxylate, which block non-selectively at least some benzodiazepine actions¹⁷⁻²². The inhibitory potency of methyl- β -carboline-3-carboxylate, an

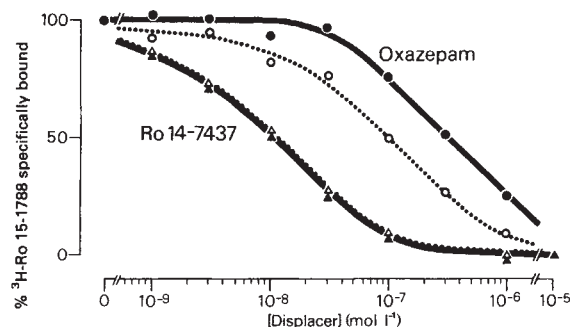


Fig. 1 Inhibition of ^3H -Ro 15-1788 binding in the presence (open symbols) and absence (solid symbols) of 100 $\mu\text{mol l}^{-1}$ GABA, by the agonist oxazepam (circles) and the antagonist Ro 14-7437 (triangles) at 35°C using freeze-thawed and extensively washed synaptic membranes⁵ of rat cerebral cortex. IC₅₀ values $\pm$ s.e.m. of at least three determinations in the absence (presence) of GABA were: flunitrazepam 27.6 $\pm$ 6 (8.2 $\pm$ 0.3 P < 0.02); diazepam 94 $\pm$ 11 (32.5 $\pm$ 5.0; P < 0.01); zopiclone 160 $\pm$ 10 (97 $\pm$ 10; P < 0.02); oxazepam 315 $\pm$ 10 (116 $\pm$ 14; P < 0.01); CL 218 872: 560 $\pm$ 30 (350 $\pm$ 20; P < 0.01). Ro 15-1788: 7.4 $\pm$ 0.8 (6.9 $\pm$ 0.5); Ro 14-7437: 13.2 $\pm$ 1.9 (14.6 $\pm$ 0.3); CGS 8216: 0.44 $\pm$ 0.1 (0.61 $\pm$ 0.1); propyl- β -CC: 11.9 $\pm$ 0.3 (11.6 $\pm$ 0.4); ethyl- β -CC 5.5 $\pm$ 0.8 (7.9 $\pm$ 0.6), all with no significant difference by Student's t -test. Methyl- β -CC, 8.8 $\pm$ 0.8 (14.8 $\pm$ 1.0; P < 0.02). Similar results were obtained at 0°C, except that the IC₅₀ values of zopiclone and methyl- β -CC remained unchanged in the presence of GABA. β -CC, β -carboline-3-carboxylate.

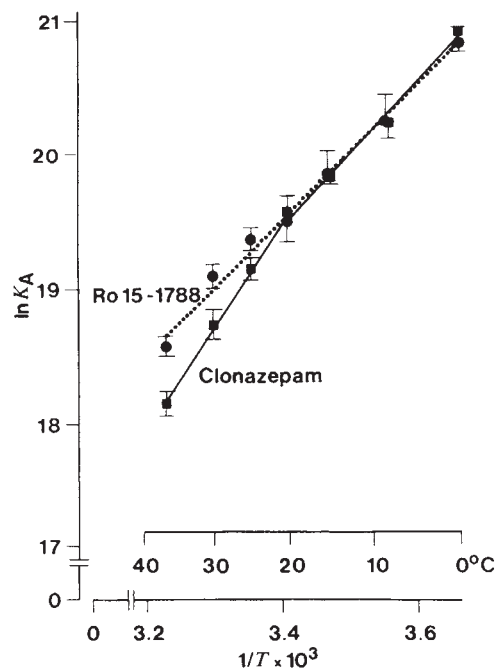


Fig. 2 Van't Hoff plot of the dependence of K_A of ^3H -Ro 15-1788 and ^3H -clonazepam binding on temperature (T), using frozen and extensively washed synaptic membranes⁵ of rat cerebral cortex. Each point represents the mean ($\pm$ s.e.m.) of three determinations. The apparent dissociation constant K_D ($1/K_A$) for ^3H -clonazepam (^3H -Ro 15-1788) at 0°C were: 0.86 $\pm$ 0.03 (0.87 $\pm$ 0.07) nmol l⁻¹; at 7.5°C: 1.68 $\pm$ 0.2 (1.72 $\pm$ 0.33); 15°C: 2.46 $\pm$ 0.12 (2.48 $\pm$ 0.41); 20°C: 3.25 $\pm$ 0.38 (3.55 $\pm$ 0.46); 25°C: 4.92 $\pm$ 0.39 (3.98 $\pm$ 0.35); 30°C: 7.46 $\pm$ 0.78 (5.16 $\pm$ 0.42); 37°C: 13.2 $\pm$ 1.3 (8.64 $\pm$ 0.57). The B_{max} values did not change with temperature. The thermodynamic parameters for ^3H -clonazepam (^3H -Ro 15-1788) binding at 37°C were: ΔG^0 = -11.20 (-11.46) kcal mol⁻¹; ΔH^0 = -14.98 (-9.87) kcal mol⁻¹; ΔS^0 = -12.20 (+5.12) EU. The parameters at 0°C were: ΔG^0 = -11.34 (-11.34) kcal mol⁻¹; ΔH^0 = -10.34 (-9.87) kcal mol⁻¹; ΔS^0 = +3.65 (+5.3) EU (entropy units).

antagonist having convulsant activity²³, was even decreased in the presence of GABA (see Fig. 1 legend). This test may be relevant not only for differentiating agonists from antagonists, but also for evaluating mixed agonist-antagonists.

The thermodynamics of the receptor binding reaction for Ro 15-1788 differ from those of clonazepam as shown by the change in standard Gibbs free energy (ΔG°), in entropy (ΔS°) and enthalpy (ΔH°) determined as described elsewhere²⁴. The van't Hoff plot, from which ΔH° is calculated, showed an inclination point at 21 °C for ³H-clonazepam binding (as for other agonists^{25,26}) but was linear for ³H-Ro 15-1788 (Fig. 2). Above 21 °C, ³H-clonazepam binding was associated with a large decrease in enthalpy which compensated for an unfavourable decrease in entropy. ³H-Ro 15-1788 binding, however, was enthalpy- as well as entropy-driven (see Fig. 2 legend). These

findings agree with a two-step model of receptor binding, as proposed for other receptors²⁴: $L + R \xrightleftharpoons{(1)} LR \xrightleftharpoons{(2)} LR'$ where L is the ligand and R the receptor. Agonists and antagonists may bind in reaction (1) with similar thermodynamic components. Due to their structural properties, agonists, but not antagonists, can participate in reaction (2) by inducing a conformational change of the receptor to R'. This conversion is thought to be associated with negative enthalpy and entropy components. Below the temperature of 21 °C, at which phase transitions of membrane lipids are known to occur, the thermodynamic parameters of ³H-clonazepam binding were almost identical to those of ³H-Ro 15-1788 binding (see Fig. 2 legend). Possibly the agonist-dependent change in BR conformation cannot be induced at temperatures <21 °C.

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Immunohistochemical mapping of vitamin D-dependent calcium-binding protein in brain

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The steroid hormone, 1, 25-dihydroxycholecalciferol (1, 25-(OH)₂D₃) causes the *de novo* synthesis of a calcium-binding protein (D-CaBP)^{1,2}. This protein is present in highest concentrations in intestine, kidney and shell gland, across which calcium is transported in relatively large amounts, but it is also found in smaller amounts in several other tissues, including brain³⁻⁷. The area of brain with the highest D-CaBP concentration is the cerebellum, where the protein is found only in the Purkinje cells^{7,8}. We have now mapped D-CaBP immunohistochemically throughout the brain of chicks and rats and present here a list of all positive nuclei. Because certain of these neurones also contain 1, 25-(OH)₂D₃ (ref. 9), we suggest that they are target cells for this hormone, thus broadening the functional significance of vitamin D to include the brain and implicating vitamin D in a more widespread action than simply a role in the calcium translocation mechanism of epithelial cells.

Intestinal D-CaBP from both birds and animals has been well characterized with respect to its molecular weight, calcium-binding constants and electrophoretic mobility^{1,10,11}. D-CaBP from chick brain resembles that of chick intestine immunologically, in molecular weight and in electrophoretic mobility and is "indeed the same protein"¹². The antisera against chick intestinal D-CaBP give precipitin lines with rat and bovine brain D-CaBP¹³ and consequently rat brain can also be stained for this protein. We have taken sections from every area of rat and chick brains and assessed them for D-CaBP-positive nuclei by an immunoperoxidase technique⁸. The protein was found in various cells throughout the brain of these two species but only

in neuronal elements and never in glial, endothelial or ependymal cells. Within any brain region it was always localized in certain specific neuronal types (Table 1 and Fig. 1a-c).

There seems to be no common factor between the D-CaBP-positive neurones listed in Table 1 that could suggest a function for this protein. However, in three locations, on the basis of electrophysiological data available, there seems to be a special need for calcium regulation. The Purkinje cells of the cerebellum^{14,15}, the neurones of the inferior olive¹⁶ and CA1 pyramidal cells of the guinea pig hippocampus¹⁷ produce voltage-dependent calcium spikes within their dendritic trees. These then induce the usual sodium-dependent action potentials from the soma-initial segment of each of these neurones. All the neurones contain D-CaBP, both in their soma and dendrites (Fig. 1a-c). The pyramidal neurones of CA3 also produce voltage-dependent calcium currents¹⁷ in their dendrites but do not contain D-CaBP. However, an important electrophysiological difference between CA1 and CA3 neurones is that although calcium currents induce bursts of spikes in both CA1 and CA3 pyramidal dendrites, in CA3 pyramids these bursts propagate to the soma, whereas in CA1 a dendritic burst produces only a single somatic spike. Perhaps D-CaBP, by regulating intracellular calcium levels, is limiting the temporal and spatial spread of calcium currents, thereby preventing or modifying bursting activity. It is thus possible that the presence of D-CaBP in a neurone is indicative of voltage-dependent calcium currents in that cell; however, this idea must be tested by intracellular recording from at least some of the cell types listed in Table 1. The pyramids of CA3, however, make it clear that calcium spikes need not imply the presence of D-CaBP. This situation may be quite common because D-CaBP was not found in dorsal root ganglia, although some ganglion cells can produce calcium spikes¹⁸.

If cells containing CaBP are to be regarded as target cells for 1,25-(OH)₂D₃, they should also contain this hormone in addition to one of the products of its action. Combining the results of this study with those^{9,19} on the autoradiographic localization of 1,25-(OH)₂D₃, which used the atlas of König and Klippel²⁰, the following sites in brain contain both the hormone and D-CaBP: amygdala, nucleus paraventricularis, nucleus paratenialis in the thalamus and some neurones in the nucleus spinalis caudalis of the nervous trigeminus. Although low uptake of labelled

Table 1 Localization of D-CaBP in brain regions of rat and chicken

Region in chicken	D-CaBP concentration		Region in rat	D-CaBP concentration
Medulla and pons				
Substantia gelatinosa trigemini	+	≡	Nucleus trigemini spinalis	++
Nucleus solitarius	+++	≡	Nucleus solitarius	++
Inferior olive	++	≡	Inferior olive	++
Nucleus magnocellularis	++	≡	Ventral cochlear nucleus	+
Nucleus subtrigeminus	++		Dorsal cochlear nucleus (scattered cells)	++
Descending vestibular nucleus (scattered cells)	+++		Nucleus of trapezoid body	+
Nucleus tangentialis	++		Dorsal nucleus of the lateral lemniscus (scattered cells)	++
Nucleus laminaris	++			
Principal trigeminal nucleus	+			
Lateral reticular formation (scattered cells)	+			
Nucleus ventralis lemniscus lateralis	+++			
Cerebellum				
Purkinje cells	+++	≡	Purkinje cells	+++
Mesencephalon				
Tectum (scattered cells)	++	≡	Superior colliculus (scattered cells)	++
Area pretectalis (scattered cells)	++	≡	Pretectal area (scattered cells)	++
Ventral tegmental area of Tsai (scattered cells)	+++	≡	Ventral tegmental area of Tsai (scattered cells)	++
Nucleus linearis caudalis	+		Central tegmental nucleus (scattered cells)	++
Nucleus intercollicularis (scattered cells)	+		Lateral tegmental nucleus (scattered cells)	++
Nucleus subceruleus ventralis (scattered cells)	+			
Nucleus mesencephalicus nervi trigemini	++			
Nucleus mesencephalicus profundus (scattered cells)	+++			
Nucleus papilliformis (scattered cells)	++			
Nucleus ectomammilaris	+			
Nucleus isthmi magnocellularis	++			
Diencephalon				
Medial habenula	+++	≡	Medial habenula	+++
Spiriformis medialis	+		Nucleus lateralis thalami, pars posterior (scattered cells)	+
Spiriformis medialis	++		Nucleus ventralis thalami (scattered cells)	+
Nucleus rotundus	+		Nucleus ventralis thalami, pars medialis	++
Nucleus triangularis	++		Nucleus reuniens	+++
Nucleus subhabenularis lateralis (scattered cells)	++		Rhomboid nucleus	++
Nucleus ventrolateralis thalami (scattered cells)	+++		Mediodorsal nucleus (scattered cells)	+
Area hypothalami posterioris (scattered cells)	++		Paraventricular nucleus of thalamus	++
Nucleus medialis hypothalami posterioris (scattered cells)	++		Parataemial nucleus of thalamus	++
Nucleus mamillaris lateralis (scattered cells)	++		Zona incerta	++
Stratum cellulare internum (scattered cells)	++		Subthalamic nucleus	++
Nucleus paraventricularis magnocellularis	+++		Lateral mammillary nucleus	+
			Medial mammillary nucleus	++
			Supra mammillary nucleus	+
			Dorsal premammillary nucleus (scattered cells)	+
			Posterior hypothalamic nucleus (scattered cells)	++
			Dorsomedial hypothalamic nucleus	+
			Area hypothalamica anterior (scattered cells)	+
			Perifornical neurones and neurones within medial forebrain bundle	++
			Supraoptic nucleus	+
			Paraventricular nucleus	++
			Arcuate nucleus (scattered cells)	+++
Telencephalon				
Paleostriatum primitivum	+		Lateral septal nucleus	+
Neostriatum and hyperstriatum (scattered cells, small nucleus)	+++		Hippocampus (granule cells of dentate gyrus and pyramidal cells of CA1)	++
			Amygdala (scattered cells)	++
			Pyiform cortex, deep cells	++
			Entorhinal cortex, deep cells	++
			Olfactory cortex, deep cells	++
Olfactory bulb, periglomerular cells	+	≡	Olfactory bulb, periglomerular cells	+++

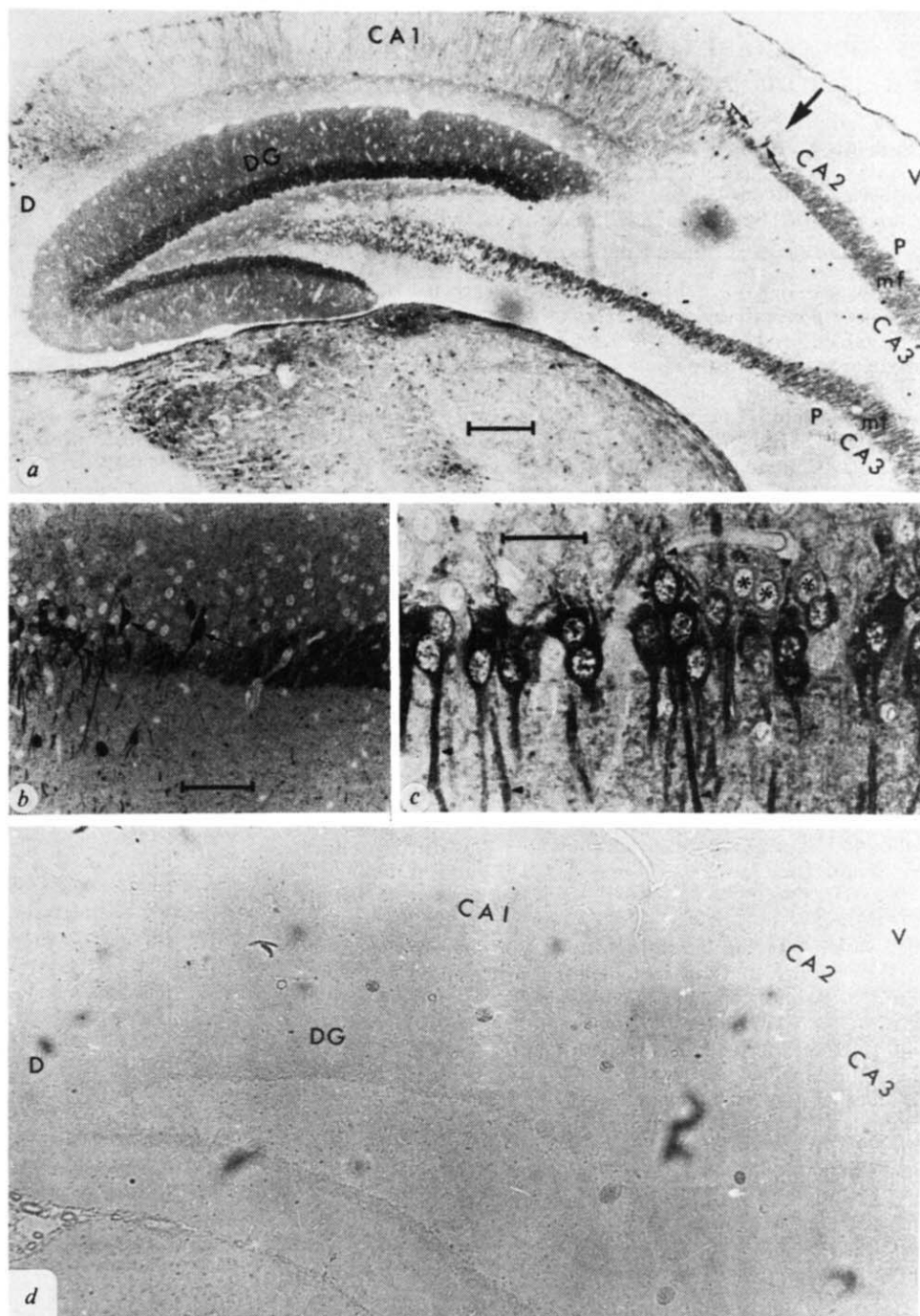
Monospecific antiserum was raised against chick duodenal D-CaBP purified to electrophoretic homogeneity by the original method of D-CaBP isolation²⁴. Brain sections (7 µm) were prepared from paraffin-embedded material after fixation with Carnoy (for rationale of fixation and further details of the technique, see ref. 8). Antiserum was used at 1:10 dilution, while goat anti-rabbit γ-globulin conjugated with peroxidase (Miles) was diluted as 1:50. Treatment with 3,3'-diaminobenzidine was for 15 min. Control sections were stained with normal rabbit serum and with supernatant from antigen and antiserum mixed in proportions of equivalence point. Other sections were stained with cresyl violet. The concentration of D-CaBP was ascertained only visually and is expressed as +, ++ and +. +++ Implies a dense reaction in the somata which was also easily visible in the dendrites, axons and their terminals⁸, while + indicates a lesser reaction in the perikaryon which may extend into the processes but was not easily detectable there. ++ Implies an intermediate situation where the reaction product was quite dark in the somata and was easily detectable in the processes but only in their proximal parts. Unless otherwise mentioned (for example, scattered cells), all neurones in each nucleus stained for D-CaBP. Those regions for which there is good comparative anatomical evidence for homology are marked ≡ whereas regions which are not known to be homologous are listed separately. Nomenclature for the brains is drawn primarily from the atlases of Karten and Hodoss²⁵, and Pellegrino *et al.*²⁶.

hormone has been demonstrated in the spinal cord, no D-CaBP was detected.

There is therefore an encouraging correlation of identity of the target cells by the two techniques, for it must be remembered that all D-CaBP-containing cells, because of the dependency of this protein on vitamin D in the intestine, must be target cells, whereas all target cells need not be positive for D-CaBP, as the synthesis of this protein may not be the response of such cells to vitamin D stimulation. However, D-CaBP is present in many

other organs^{5,6} and in some cases the cell types have been identified^{8,21}. Evidence for the view that a wide range of tissues have target cells for 1,25-(OH)₂D₃ is provided by the autoradiographic localization of the hormone in cells which also contain D-CaBP^{9,19,22}, only a few of which occur in the central nervous system. Initial studies with epithelial tissues such as the intestine and shell gland have connected the function of D-CaBP with transcellular calcium transport, as seen in calcium absorption or egg shell formation²³. This view is clearly not

Fig. 1 *a-c*, Transverse sections of rat hippocampus, stained with antiserum. In low power view (*a*) the dark reaction product indicating the presence of D-CaBP is seen in discrete sites. The blank areas serve as internal controls. For orientation, D and V denote dorsal and ventral sides of the brain. The D-CaBP is seen in the granule cells of the dentate gyrus (DG) and their mossy fibre efferents (mf). Pyramidal cells (small arrows) within CA1 are also positive. *b* Shows a higher magnification of the area about the large arrow in *a*, illustrating the abrupt transition between CA1 and CA2. Note that whereas pyramidal cells of CA1 (small arrows) are positive, pyramidal cells of CA2 as well as CA3 (P in *a*) and their Schaeffer collaterals within CA1 are devoid of D-CaBP. *c*, A further higher magnification view of CA1 shows the reaction product in both apical and basilar dendrites (arrow heads) of CA1 pyramidal cells. Note that some neurones do not show any reaction product (asterisk). It is not known whether these are pyramidal cells or another type of cell (for example, basket cells). Scale bars in *a*, *b* and *c* represent 300, 100 and 40 μ m respectively. *d*, Control, similar to *a* but from section stained with normal rabbit serum.



supported by the presence of D-CaBP in so many cell types uninvolved in such processes. It may be that D-CaBP is involved in more than one physiological function, in which case it might have a single molecular action which has been incorporated into

a variety of processes. The latter almost certainly involve the membrane transport of calcium but whether the protein is involved at this point or in the subsequent need to regulate intracellular calcium levels awaits further study.

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Evidence that types I and II interferons have different receptors

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Interferons (IFNs) are a class of proteins, secreted by animal cells in response to various inducers¹, which confer resistance to viral infections and are designated according to their cellular origin or to the inducing agent. Viruses induce type I interferon, subdivided into α -interferon, produced by leukocytes (Le) or lymphoblastoid (Ly) cells, and β -interferon, produced by fibroblasts¹. Mitogens and antigenic stimuli induce in lymphocytes type II immune IFN- γ . Interferons seem to bind to specific receptors² to elicit a variety of cellular responses³; several early studies provided indirect evidence for such binding⁴⁻⁶. Direct evidence for specific interferon receptors was presented by Aguet⁷, who showed that biologically active ¹²⁵I-labelled mouse (Mu)IFN binds to sensitive L1210-S cells, but not to interferon-resistant L1210-R cells⁸. The main obstacle in carrying out such studies is the availability of pure interferon. Recently, Maeda *et al.*⁹ constructed plasmids containing human interferon sequences. The plasmid p104 was used as a probe to isolate the coding region of a human interferon (IFLrA), which was expressed in *Escherichia coli*¹⁰ and purified with monoclonal antibodies¹¹. This interferon, designated HuIFN- α A, was labelled with ¹²⁵I for binding assays on human cells. We have determined the specificity of different HuIFNs for the cellular sites which bind HuIFN- α A and show here that HuIFN- γ does not compete for binding, whereas all type I HuIFNs do.

The HuIFN- α A was iodinated in conditions designed to minimize the concentration of H₂O₂ in the reaction (Fig. 1). The high stability of the interferon allowed prolonged incubation and resulted in retention of biological activity. The ¹²⁵I-labelled HuIFN- α A had a specific activity of ~50 mCi per mg and an antiviral titre in different experiments of 50–75% of the unreacted interferon, when assayed within a week after iodination. The HuIFN- α A was iodinated in ~15% of the tyrosine residues, as determined from the specific activity of ¹²⁵I and the recovery of labelled protein. As there are four tyrosines per HuIFN- α A molecule¹², at most 60% of the molecules were mono-iodinated. We have as yet no information on the proportion of molecules having different degrees of iodination.

The product of these iodination reactions showed a major molecular weight (*M_r*) component of ~20,000 (as calculated from the DNA sequence¹², HuIFN- α A has a *M_r* of 19,390) and traces of other components, when analysed by gel electrophoresis (Fig. 1). This preparation was further fractionated by chromatography on Sephadex G-75 and the fractions analysed by gel electrophoresis and for antiviral activity (Fig. 1). A single peak of antiviral activity was eluted, corresponding to the major peak of radioactive protein. This peak was well separated from iodinated bovine serum albumin (BSA), which represented the major protein contaminant. Some carrier BSA was presumably iodinated when added after inhibiting the reaction with NaN₃ (see Fig. 1 legend). The fractions containing ¹²⁵I-HuIFN- α A were combined and used in subsequent experiments.

We investigated the interaction of ¹²⁵I-HuIFN- α A with cellular binding sites by incubating it with suspensions of human cells in tissue culture. At the end of the incubation, the cells were centrifuged through two sucrose layers¹³ and the radioactivity sedimenting with the cell pellet was measured (Table 1). Greater binding was obtained with Daudi cells than with the other lymphoblastoid cells tested or with HeLa cells. Therefore, we used Daudi cells in all further experiments. Even with these cells, relatively small amounts of ¹²⁵I-HuIFN- α A were bound and the assays were carried out with 4–10 ml of cell suspension.

Binding was extremely reproducible: each preparation of labelled interferon gave essentially identical results when tested in standard conditions for several weeks, despite some decline in antiviral activity.

The time course of binding at 0 °C and 37 °C is shown in Fig. 2a. There was very little binding at 0 °C, whereas at 37 °C substantial binding occurred which reached a plateau after ~1 h. All subsequent experiments were carried out at 37 °C, the temperature of the cell cultures. ¹²⁵I-HuIFN- α A binding approached saturation when increasing concentrations were incubated with a standard cell aliquot; a Scatchard analysis of these data gave an apparent single affinity constant of 7×10^9 M⁻¹ (Fig. 2b). This binding was abolished when large amounts of HuIFN- α (Le) were included in the incubations (Fig. 2b). These results indicate that the binding involves specific cellular sites. The number of binding sites per cell, calculated from the intercept with the abscissa in the Scatchard plot¹⁴, is ~5,000. These observations are consistent with the binding of ¹²⁵I-HuIFN- α A to cellular receptors, although the identification of a specific receptor would require its characterization as a macromolecular complex with interferon or its purification. For

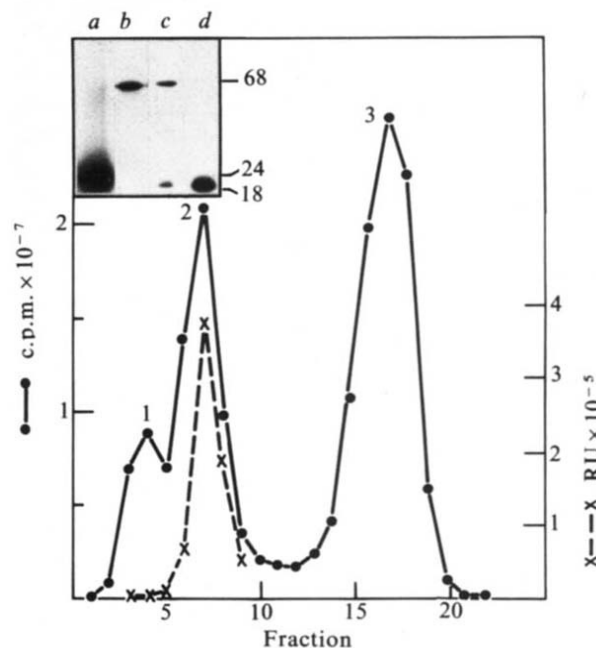


Fig. 1 Chromatography of iodinated interferon on Sephadex G-75. The HuIFN- α A was purified by Dr Sidney Pestka (Roche Institute of Molecular Biology) on an affinity column of monoclonal antibodies to a specific activity of 2×10^8 U per mg. It was iodinated in a reaction containing in 0.1 ml: 2.2 μ g HuIFN- α A, 12.5 μ M Na¹²⁵I (2.5 mCi, ~2,000 Ci mmol⁻¹), 10 μ l of hydrated Enzymobead iodination reagent (Bio-Rad) containing immobilized glucose oxidase and lactoperoxidase, 0.3% β -D-glucose and phosphate-buffered saline (PBS) pH 7.0. The reaction was stopped after 24 h by the addition of 0.2 M NaN₃. After 15 min, 0.2 mg of BSA in 0.1 ml PBS was added and the sample applied to a 2.5×0.7 cm column of Sephadex G-10 equilibrated with PBS containing 1 mg ml⁻¹ BSA (buffer A). This column was washed with 1 ml of buffer A and the eluate applied to a 25×0.7 cm column of Sephadex G-75 equilibrated with buffer A. The column was eluted at a flow rate of 6 ml h⁻¹ and 0.44-ml fractions were collected. All these operations were carried out at room temperature. The column fractions were stored at 4 °C and analysed for radioactivity (●) and antiviral activity (×). The latter was measured by the protection of monolayers of the human lung carcinoma cell line A549 from infection with encephalomyocarditis virus. Monolayers in multi-well plates were infected with 4×10^5 plaque-forming units of virus and the cytopathic effect quantitated by a viral dye uptake method²². The HuIFN- β standard (G-023-902-527, obtained from NIH) was used as a reference and the antiviral titre is expressed in NIH reference units (RU). Column fractions were also analysed by electrophoresis on 15% polyacrylamide slab gels²³. The autoradiographs are shown in the top left corner: a, unfractionated ¹²⁵I-HuIFN- α A; b, peak 1; c, fraction 5; d, peak 2. About 10,000 c.p.m. of each fraction and 100,000 c.p.m. of unfractionated interferon were analysed. The molecular weight (in thousands) of protein standards run in parallel is indicated at the right-hand side: 68, BSA; 24, trypsinogen; and 18, β -lactoglobulin. Peak 3 is free ¹²⁵I.

Table 1 Binding of ^{125}I -labelled interferon to human cells

Cell line	^{125}I -HuIFN- αA bound (c.p.m.)
Daudi	880
Raji	270
Molt-4	260
HeLa	310

The lymphoblastoid cells were grown in stationary cultures or in roller bottles in Dulbecco's medium with 10% horse serum; HeLa cells were grown in spinner cultures in Eagle's medium supplemented with 7.5% calf serum. Each assay contained in 5 ml of culture medium: 2.5×10^6 cells and 2 ng of ^{125}I -HuIFN- αA ($\sim 60,000$ c.p.m.). After 1 h at 37°C the samples were applied over two 4-ml layers of ice-cold 5 and 10% sucrose in buffer A and centrifuged at $20,000g$ for 2 min¹³. The pellet obtained was counted in a γ -counter.

convenience, the cellular binding sites for HuIFN- αA are referred to as receptors.

The observation that unlabelled interferon abolished the binding of ^{125}I -HuIFN- αA led us to assay different interferons in competition experiments (Fig. 3). The HuIFNs used differed widely in purity, and antiviral activities had been measured in different laboratories. We determined the antiviral titre using the standard assay described in Fig. 1 legend. The competition with unlabelled HuIFN- αA could be superimposed on that obtained with HuIFN- $\alpha(\text{Ly})$, whereas HuIFN- $\alpha(\text{Le})$ competed slightly less effectively (Fig. 3a). HuIFN- β preparations of quite different purity gave superimposable competition curves, indicating that other proteins present in the preparations do not interfere in this assay (Fig. 3b). Finally, HuIFN- γ and MuIFN- β , which is inactive on human cells¹⁵, competed only poorly, if at all (Fig. 3b). The competition with different HuIFN- β samples could not be explained by the presence of contaminating HuIFN- α in the preparations. This was established by pre-incubating HuIFN- β and HuIFN- $\alpha(\text{Le})$ with an appropriate amount of anti-HuIFN- $\alpha(\text{Le})$ antiserum, which does not significantly cross-react with HuIFN- β , as previously described¹⁶. This resulted in decreased competition of the HuIFN- $\alpha(\text{Le})$, but had no effect on HuIFN- β competition (see Fig. 3 legend). It seems, therefore, that all type I HuIFNs compete with ^{125}I -HuIFN- αA for binding to the same receptors.

These results show that HuIFN- γ does not bind with high affinity to HuIFN- αA receptors and indicate that type II interferon may interact with different receptors. Ankel *et al.*¹⁷ proposed that there are two distinct classes of interferon-binding

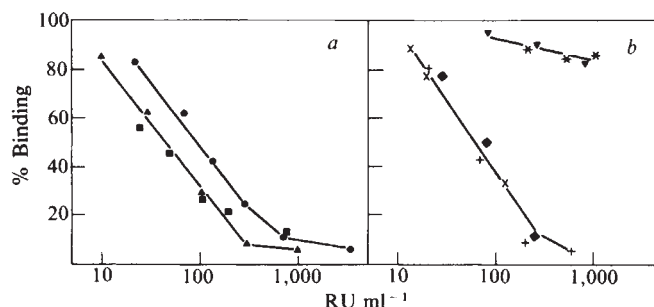


Fig. 3 Competition of different interferons with ^{125}I -HuIFN- αA for binding to Daudi cells. Incubations were as described in Fig. 2a, with the addition of the indicated concentrations of the following interferons: HuIFN- $\alpha(\text{Le})$ (●; from Dr K. Cantell), HuIFN- $\alpha(\text{Ly})$ (▲; from Dr C. B. Anfinsen), unlabelled HuIFN- αA (■), highly purified ($>10^8$ RU per mg) HuIFN- β (◆; from Dr E. Knight Jr), two less purified samples of HuIFN- β (×; from Dr J. Horosiewicz, 10^6 RU per mg; and +; from the Interferon Working Group of the NCI, 3×10^5 RU per mg), HuIFN- γ (▼), purified as described elsewhere²⁴, was given by Dr J. Vilček and Dr Y. K. Yip, and MuIFN- β (*) was a gift from Dr Peter Lengyel (this preparation was purified to $>10^8$ RU per mg (ref. 15) and was neutralized using anti-MuIFN- β antiserum²⁵ in Dr Lengyel's laboratory). The results are plotted as % binding relative to an assay with no added competitor. As a control for the specificity of the competition, 2,000 RU of HuIFN- $\alpha(\text{Le})$ or HuIFN- β in 0.1 ml were incubated with 20 μl anti-HuIFN- $\alpha(\text{Le})$ antiserum at 37°C for 30 min and then overnight at 0°C , as described elsewhere¹⁶. These samples were tested in competition assays. The binding with HuIFN- β was 11%, unchanged relative to a sample not treated with antiserum, whereas that with HuIFN- $\alpha(\text{Le})$ increased from 10 to 34%.

sites in mouse cells, each specific for one interferon type, on the basis of experiments with L1210-R cells selected for resistance to Type I MuIFN⁸; these cells responded to the antiviral and anti-growth activities of type II interferon¹⁷. Moreover, gangliosides, which may be part of type I interferon receptors⁵, inhibit the activity of type I but not that of type II interferon¹⁷. Distinct receptors for the two interferons may account for the different pathways of induction of the 2'5'-oligo(A) polymerase elsewhere reported¹⁸ and possibly for the potentiation of activity of one interferon type by simultaneous treatment with the other¹⁹. Interferons of the same type, however, do not potentiate each other but show additive effects¹⁶, as expected if they interact with the same receptor.

In these studies we measured interferon binding at 37°C . At this temperature interferon may interact with cellular receptors in a complex way, possibly as a result of a series of metabolic events. This was suggested by the observations of Friedman⁴, who treated chick fibroblasts with interferon at 4°C . Digestion with trypsin at this temperature inhibited the development of the antiviral state when the cells were shifted to 37°C . A short incubation at 37°C , however, apparently rendered the interferon trypsin resistant. This led Friedman⁴ to postulate that interferon binding at 4°C involves cell-surface sites accessible to trypsin. Aguet⁷ previously reported that binding of ^{125}I -labelled MuIFN to L1210-S cells is markedly higher at 37°C than at 4°C ; in this study an apparent dissociation constant (K_d) of $1-2 \times 10^{-11}$ M was estimated from the interferon concentration required to reach half-saturation of binding sites. From our data we calculate a K_d of $\sim 1.5 \times 10^{-10}$ M. These K_d values may reflect the different specific activities of the interferons: 1×10^9 U per mg protein estimated by Aguet⁷ for the MuIFN and 2×10^8 U per mg for the HuIFN- αA . The biological significance of these differences in specific activity remains to be established.

Recent advances, in the study of many polypeptide hormones have provided guidelines for investigating their interaction with cellular receptors^{20,21}. Interferons may be regarded as polypeptide hormones because of their role in communicating from cell to cell a specific set of instructions, which leads to a wide variety of effects³. These studies provide an initial characterization of a HuIFN-receptor interaction, and are potentially

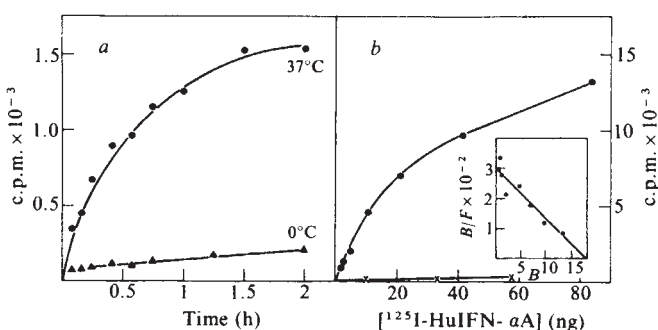


Fig. 2 Time course of binding of ^{125}I -labelled interferon (a) and saturation of binding sites (b). For each time point in a, 8×10^6 Daudi cells were incubated in 4 ml at the indicated temperature with 1 ng ^{125}I -HuIFN- αA , as described in Table 1. In b, 5×10^6 Daudi cells in 10 ml were incubated at 37°C for 90 min with the indicated amount of ^{125}I -HuIFN- αA ; the c.p.m. bound per assay are indicated (●). Binding in the presence of 4×10^3 units of HuIFN- $\alpha(\text{Le})$ per ng HuIFN- αA was also determined (×). Inset shows a Scatchard plot of the data in b; B and F are concentrations of bound and free ^{125}I -HuIFN- αA (c.p.m. $\times 10^{-3}$). The samples were centrifuged through two sucrose layers of 9 ml each, as described in Table 1 legend.

useful in studying the role of interferons as regulators of cellular functions.

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Type C virus particles in a cord T-cell line derived by co-cultivating normal human cord leukocytes and human leukaemic T cells

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A recent nationwide survey of the lymphocyte subpopulations of leukaemia and lymphoma in Japan has disclosed a high incidence of adult T-cell leukaemia (ATL)¹. One of the striking features of this disease is the clustering of patients in the southwestern part of Japan^{1,2}. We have established a continuous culture line of leukaemic T cells from a patient with ATL^{3,4}. This cell line, MT-1, was found to carry type C virus particles and ATL-associated antigens (ATLA) which specifically reacted with sera from all ATL patients and about 25% of healthy adults in the endemic area⁵. We report here that by co-culture of ATL cells from a female patient and normal human cord leukocytes from a male infant, a T-cell line of cord leukocyte origin was established. Numerous type C virus particles as well as ATLA were detected in this cell line.

A 45-yr-old woman was admitted to the hospital with generalized lymphadenopathy and hepatosplenomegaly. Her blood leukocyte count was 207,300 per mm³, with 93% abnormal lymphoid cells having indented or lobulated nuclei. Most of these cells formed spontaneous sheep erythrocyte rosettes. Peripheral leukocytes separated by Ficoll-Conray gradient centrifugation were cultured in 35-mm Petri dishes (3 ml per dish) with RPMI 1640 medium supplemented with 10% human cord serum, 10% fetal calf serum and antibiotics. The initial cell density was adjusted to 5 × 10⁶ per ml. The

cultures were incubated at 37 °C in a humidified 7.5% CO₂ atmosphere and fed twice a week. After 2 months of culture, the number of cells decreased considerably and the cells were co-cultured with umbilical cord leukocytes (1 × 10⁶ per ml) from a male infant, with the hope of stimulating the growth of ATL cells. After 2 months of co-culture, a lymphoid cell line was established that was transferable every 3–5 days. This cell line, MT-2, grew in suspension forming clumps of cells and has been maintained in continuous culture for 1.5 yr. No T-cell growth factor was used. Unexpectedly, cytogenetic studies on MT-2 showed a normal male karyotype and its cord leukocyte origin was determined. MT-2 cells expressed complement receptors and Ia antigens in addition to having sheep erythrocyte receptors and T-cell membrane antigens. The cultured cells were negative for Epstein-Barr virus nuclear antigen. The establishment and characteristics of this cell line have been briefly reported elsewhere⁶.

The acetone-fixed cells were examined for ATLA by indirect immunofluorescence using ATL patients' sera and fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG as previously described⁵. The MT-2 line gave a positive reaction with three reference sera from ATL patients but not with three negative control sera. Almost 100% of cells showed brilliant staining in the periphery of the cytoplasm at a serum dilution of 1:10 (Fig. 1). Unfixed MT-2 cells were similarly stained by the indirect immunofluorescence method and almost all the cells exhibited surface membrane fluorescence with the positive reference sera. These reference sera, however, did not react with various human haematopoietic cell lines, including six T-cell lines (HPB-ALT, HPB-ALL, TALL-1, RPMI 8402, Molt-4 and CCRF-CEM), four B-cell lines (Raji, Wil-2, TL-1 and BALL-1) and four non-T non-B cell lines (HL-60, NALL-1, HPB-NUL and K-562). Of these, TALL-1, BALL-1 and NALL-1 are acute lymphoblastic leukaemia cell lines established by us and maintained in our laboratory in the same conditions as used for MT-2. This would exclude the possibility that some serum component(s) or other exogenous products in the culture medium elicited the immunofluorescent reaction. Thin-section electron microscopy of MT-2 cells revealed numerous type C virus particles in the extracellular spaces (Fig. 2). The virus particles which were predominantly mature were between 85 and 145 nm in diameter. The mature virus particles consisted of an electron-dense nucleoid and an outer membrane. The immature virus particles were doughnut-shaped with an electron-luscent centre. Virus particles budding from the cell membrane were rarely observed.

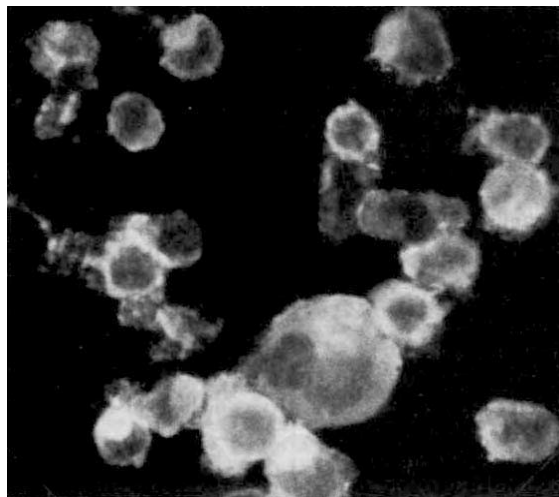


Fig. 1 Immunofluorescence micrograph of acetone-fixed MT-2 cells showing ATLA in the cytoplasm with brighter fluorescence in the periphery. All the cells, including giant cells, are fluorescent. The cells were first reacted with serum from an ATL patient and then incubated with FITC-conjugated goat anti-human IgG. ×750.

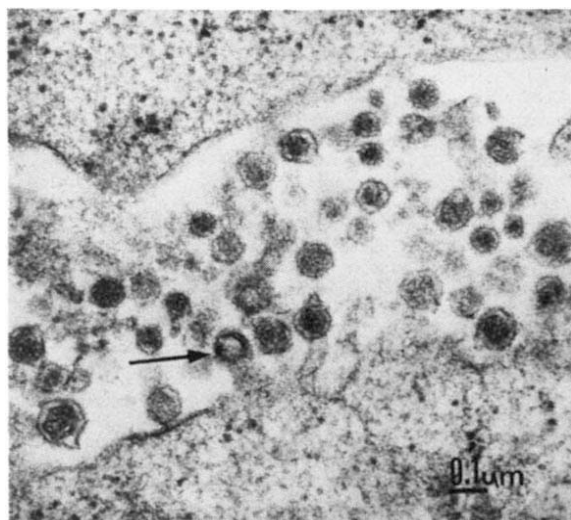


Fig. 2 Electron micrograph of MT-2 showing numerous type C virus particles in the extracellular space. One particle is budding from the cell membrane (arrow). The cells were fixed in 3% glutaraldehyde and 1% osmium tetroxide and embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate. $\times 59,000$.

Fresh ATL cells from the patients do not express ATLA; this antigen expression and type C virus production occur spontaneously after several days of *in vitro* culture (Y.H., unpublished observation). This is consistent with persistent association of ATLA and type C virus particles in a cultured ATL cell line MT-1 (ref. 5). It is postulated, therefore, that during co-culture of ATL cells and normal human cord leukocytes, a type C virus or its genome was transmitted from ATL cells to cord T cells by extracellular or cell-to-cell infection and that the infected T cells were transformed into the virus-producer cell line MT-2. The presence of exclusively ATLA-positive cells and numerous virus particles in MT-2 is indeed significant because MT-1 contained only a few per cent of ATLA-positive cells and rare virus particles⁵. Consistent with the present report, we have recently characterized another T-cell line derived from cord blood lymphocytes by co-cultivation with ATL cells from a different patient. This cell line also harbours abundant type C virus particles and ATLA (manuscript in preparation). Contamination of our cells by animal oncoviruses is unlikely, as neither these viruses nor murine cells have been handled in our laboratory for several years. According to Barbacid *et al.*⁷, human sera recognized glycoproteins of animal oncoviruses grown in rodent or carnivore cells but not those of the same viruses grown in human cells.

The present results strongly suggest that the type C virus we have detected in our cell lines is aetiologically related to ATL. Recently, Poesz *et al.*⁸ also isolated a type C retrovirus in a T-cell line derived from a patient with cutaneous T-cell lymphoma (mycosis fungoides). Thus, these independent observations in the United States and Japan lead us to think that at least certain T-cell malignancies may be caused by a closely related, if not identical, viral agent.

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Rous sarcoma virus transforming protein, p60^{src}, expressed in *E. coli*, functions as a protein kinase

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The product of the Rous sarcoma virus (RSV) transforming gene, *src*, is a phosphoprotein of molecular weight (M_r) 60,000 (pp60^{src})^{1–3} that is responsible for cell transformation as well as fibrosarcoma formation in a variety of animals^{4,5}. Several experiments suggest that pp60^{src} is a protein kinase^{6–10} with the unusual capacity of phosphorylating tyrosine residues^{11,12}, but additional evidence would be of value. Earlier studies had suggested that bacteria were unable to carry out protein phosphorylation¹³, although more recent findings indicate that distinct protein kinases and phosphatases are present in *Salmonella typhimurium*¹⁴. Despite this observation, because of the evolutionary distance, the enzymatic activities observed in bacteria are likely to be carried out by proteins unrelated to those expressed in eukaryotic cells. Thus, to study the RSV transforming protein synthesized in the absence of the variety of protein kinases expressed in normal host cells (for reviews see refs 15, 16), we have now constructed plasmids that express p60^{src} in *Escherichia coli*. Analysis of p60^{src} produced in *E. coli* and selected by immunoaffinity chromatography, shows that it has the capacity to phosphorylate proteins at tyrosine residues; this activity is specifically inhibited by anti-p60^{src} IgG. Extracts from *E. coli* carrying identical plasmids but lacking the *src* gene yield no detectable enzyme activity. These data, taken with those previously published^{9,10,17}, lead to the conclusion that the RSV *src* gene encodes a protein kinase.

Bacteria carrying recombinant molecular clones were radiolabelled in culture with ³⁵SO₄²⁻ and the cellular extracts subjected to immunoaffinity column chromatography as described in Fig. 1 legend. The presence of p60 among the proteins eluted from the columns was confirmed by phosphorylation of the preparations *in vitro* with the catalytic subunit of the cyclic AMP-dependent protein kinase (CAT), a previously characterized post-translational modification of pp60^{src} (ref. 18). As shown in Fig. 1, lysates prepared from bacteria carrying a *lac-src* fusion gene in pBR325 yielded both a ³⁵S-labelled protein of M_r 60,000 and a protein of the same molecular weight that could be phosphorylated by CAT, whereas lysates prepared in exactly the same way from cells carrying only *lac* UV5 DNA in pBR325 yielded no detectable protein of a similar nature.

To determine whether p60^{src} prepared from bacteria as described above functioned in a manner similar to pp60^{src} prepared from eukaryotic cells, protein kinase reactions were carried out and the results are presented in Fig. 2. To reduce the possibility that any activity detected originated in the protein substrates or the IgG, these were incubated at 60 °C for 5 min before use. The results show that the extract prepared from the *lac* UV5-pBR325 bacteria yielded no detectable phosphotransferase activity when assayed alone or with α and β tubulin or casein as potential substrates (Fig. 2, tracks 1, 2, 3 [*lac*]). In contrast, an equal quantity of extract prepared from the *lac-src* bacteria yielded readily demonstrable phosphorylation of α and β tubulin and casein (Fig. 2, tracks 1, 2, 3 [*lac-src*]). These proteins have been shown to be efficiently phosphorylated by pp60^{src} prepared from eukaryotic cells^{10,12,17}. Moreover, the phosphorylation of casein was inhibited by the prior addition of anti-p60^{src} IgG but not IgG from a non-immune rabbit (tracks 4, 5). Note that anti-p60^{src}

Fig. 1 Polyacrylamide gel electrophoretic analysis of bacterial extracts after immunoaffinity chromatography. The *lac* UV5 promoter-operator 203-base pair (bp) fragment was constructed by F. Fuller and its use as a 'portable promoter' was described previously²¹. The DNA fragment (obtained from David L. Hare and John R. Sadler of this institution) contains an *Eco*RI site 65 bp from the start point of transcription and encodes nine amino acids of the *lacZ* gene. By the use of *Eco*RI linkers this promoter was fused to the 5' end of the *src* gene previously cloned from RSV-infected cells²². *E. coli* expressing p60 was detected by immunoprecipitation of ³⁵S-labelled extracts from cells containing the *lac* UV5 promoter-operator as described previously²⁰, and one of these clones was used for the studies described here. *E. coli* carrying the *lac* UV5 promoter in pBR325 in the same orientation as in the *src*-containing clone served as a control for the expression of p60. DNA sequence analysis and restriction nuclease mapping of the cloned DNA indicated that the primary translation product under control of the *lac* promoter should contain the entire *src* gene product plus 11 additional amino acids at the NH₂ terminus encoded by the *lacZ* gene and the linkers used to construct the plasmid. Peptide mapping of the p60 produced in *E. coli* supported this interpretation. Bacteria were grown in M9 minimal medium (6 g Na₂HPO₄, 3 g KH₂PO₄, 0.5 g NaCl, 1 g NH₄Cl per l) containing 0.8% glucose, 0.4% casamino acids, 2 µg ml⁻¹ thiamine, 40 µM MgSO₄, 0.1 mM CaCl₂, 1 mM isopropyl-β-D-thiogalactopyranoside and 2.5 µCi ml⁻¹ H₂³⁵SO₄ (NEN) to an A₅₅₀ of 1.5, collected by centrifugation, washed once in phosphate-buffered saline and frozen quickly in liquid nitrogen. Frozen pellets corresponding to about 1 l of cells were prepared for immunoaffinity chromatography by suspension in 14 ml of 10 mM Tris, 20% sucrose and 1.5 mg ml⁻¹ lysozyme. After 5 min at 4 °C DNase was added to a final concentration of 60 µg ml⁻¹ and incubation continued at 4 °C for another 5 min. The lysate was brought to a final volume of 20 ml with detergent-containing buffer at a final concentration of 1% NP40, 0.5% sodium deoxycholate, 150 mM NaCl, 5 mM dithiothreitol, 10 mM Tris pH 7.2. Lysates were clarified at 10,000g for 30 min; the protein concentration at this point was about 10 mg ml⁻¹. The 10,000g supernatant was subjected to immunoaffinity chromatography as described previously⁹. Briefly, serum from a tumour-bearing rabbit selected to immunoprecipitate p60^{src} encoded by the Prague A strain of RSV (the strain of the original source of the DNA) was used to prepare IgG and, subsequently, an immunoaffinity matrix at a concentration of 7 mg IgG per ml of gel. About 140 mg of protein was applied to a 6-ml column and washed as described previously except that the RIPA wash was omitted and the protein adsorbed to the column was eluted with 0.75 M KSCN. The elution of protein was monitored by liquid scintillation spectrometry of a sample of each fraction and the peak fractions were immediately dialysed against 50% glycerol containing 1 mM EDTA, 20 mM phosphate pH 7.2 and 5 mM dithiothreitol followed by dialysis against 10% glycerol and again 50% glycerol in the same buffer to achieve about a 10-fold concentration of protein. Quantitation of the protein yield is not significant because the immunoaffinity columns are overloaded in the conditions used here. Polyacrylamide gel electrophoresis was carried out as described previously⁹. ³⁵S-labelled proteins were visualized by fluorography²³ and ³²P-labelled proteins by autoradiography with the aid of Dupont Lightning Plus intensifying screens. Left panel: Proteins purified by immunoaffinity chromatography of lysates prepared from bacteria labelled in culture with ³⁵SO₄²⁻. Track 1, *lac* pBR325; 2, *lac*-*src* pBR325. Right panel: The preparations depicted in the left panel were incubated with CAT in the presence of 5 mM MgCl₂ and 1 µM [γ-³²P]ATP before analysis. Track 1, *lac* pBR325; 2, *lac*-*src* pBR325. The catalytic subunit of cyclic AMP-dependent protein kinase (CAT) was purified as described²⁴ and was a gift of James L. Maller.

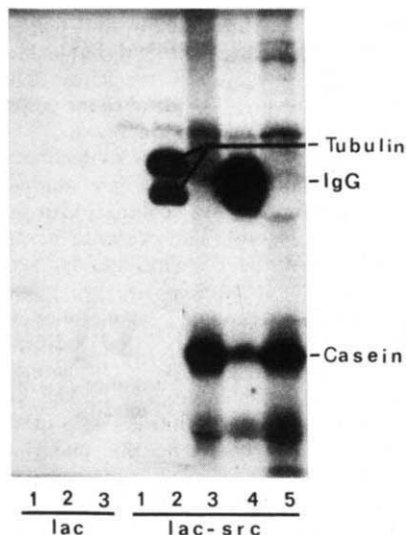
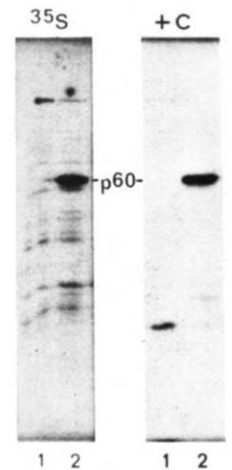


Fig. 2 Phosphorylation of various proteins by p60^{src} partially purified from *E. coli*. 10 µl (10 ng) of the preparations described in Fig. 1 were incubated at room temperature for 10 min with the indicated additions in a total volume of 25 µl. MgCl₂ and [γ-³²P]ATP (1,000–6,000 Ci mmol⁻¹) were added to a final concentration of 5 mM and 1 µM, respectively, and incubation was continued for 30 min at room temperature. Proteins were then resolved by polyacrylamide gel electrophoresis and autoradiography. Left side (*lac*): Track 1, *lac* pBR325 alone; 2, plus α and β tubulin; 3, plus casein. Right side (*lac*-*src*): Track 1, *lac*-*src* pBR325 alone; 2, plus α and β tubulin; 3, plus casein; 4, plus casein and anti-p60^{src} IgG; 5, plus casein and non-immune IgG. Casein was present at 1 mg ml⁻¹, the IgGs at 350 µg ml⁻¹ and tubulin at 100 µg ml⁻¹. Tubulin was prepared from rabbit brain as described previously²⁵ and was heated at 80 °C for 5 min before use.

IgG is phosphorylated in the reaction, as is the case when pp60^{src} from eukaryotic cells is similarly analysed^{9,10}.

The protein kinase activity associated with pp60^{src} purified from eukaryotic cells is specific for tyrosine residues in IgG¹¹ and several other protein substrates^{12,17}. To establish further the relationship between the activity described above and that previously characterized, phosphoamino acid analysis was carried out on the products shown in Fig. 2. As seen in Fig. 3, the major amino acid phosphorylated in α and β tubulin and in casein was tyrosine.

We describe here our preliminary characterization of the functional activity of the RSV *src* gene product expressed in *E. coli* and show that it has the capacity to phosphorylate tyrosine residues in protein substrates. Moreover, the phosphotransferase activity is inhibited by anti-p60^{src} IgG but not non-immune IgG and results in the phosphorylation of anti-p60^{src} IgG. These three characteristics, (1) immune IgG phosphorylation, (2) tyrosine specificity and (3) specific inhibition of the phosphorylation of exogenous substrates, all demonstrate the *src*-gene-specific nature of the enzymatic activity observed. No protein kinase activity is detectable in immunoaffinity purified preparations from identical amounts of bacteria that lack the *src* gene. Although the fusion protein studied here contains 11 additional amino acids (see Fig. 1 legend), we had anticipated that small changes at the amino terminus of p60^{src} would not seriously interfere with its function because p60^{src} shows a remarkable plasticity at the NH₂ terminus while still functioning to transform cells. This is most dramatically shown in studies of RSV recovered after passage of virus with partial *src* gene deletions through chickens, which show that the recovered viruses encode *src* gene products that may be 2–3,000 daltons longer or shorter at their NH₂ termini but that still result in cell transformation and tumour formation¹⁹.

The expression of the p60^{src}-specific protein kinase activity in *E. coli* strongly supports the previous conclusion that the enzymatic activity observed in preparations from eukaryotic cells is actually encoded in the *src* gene and is not the result of the co-purification of one of the many cell-encoded protein kinases expressed in RSV-infected cells. Although we estimate that the p60^{src} from *E. coli* is about 10% as active as a similar preparation from eukaryotic cells, the results described here should be regarded as qualitative because we anticipate that modification by phosphorylation with, for example, purified

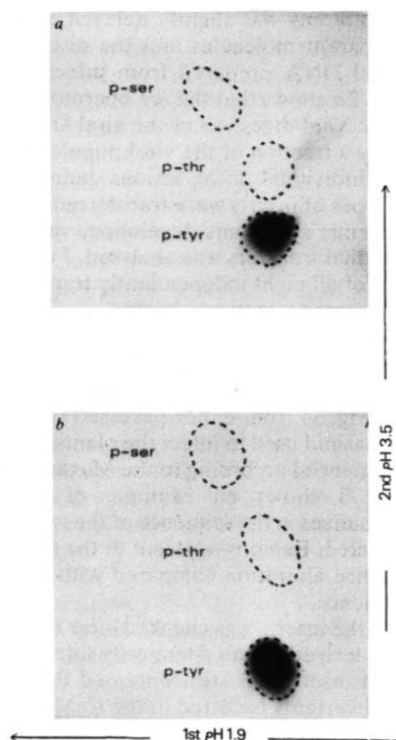


Fig. 3 Phosphoamino acid analysis of α and β tubulin and casein phosphorylated by $p60^{src}$. Phosphorylated α and β tubulin and casein, as shown in Fig. 2, right, tracks 2 and 3, were eluted from a preparative gel, precipitated and hydrolysed in 6 M HCl at 100 °C for 3 h. The phosphoamino acids were resolved by two-dimensional electrophoresis at pH 1.9 and 3.5 and visualized by autoradiography as described previously¹². Authentic phosphoamino acids were included in the sample and were visualized by ninhydrin staining. *a*, α and β tubulin; *b*, casein.

CAT may affect the activity. The evolutionary distance of *E. coli* from the normal RSV hosts makes it unlikely that they would harbour protein kinases such as the cyclic AMP-dependent protein kinase known to modify $p60^{src}$ (ref. 18). We have not detected phosphorylation of $p60^{src}$ in *E. coli*²⁰ and, if additional experiments confirm that the protein is unphosphorylated in *E. coli*, $p60^{src}$ prepared in this manner should prove useful for the study of the effect of phosphorylation on its function. Similarly, analysis of the *src* gene product produced in *E. coli* should be useful for the resolution of the issue of whether $pp60^{src}$ has the capacity to phosphorylate itself during *in vitro* phosphotransferase reactions, an activity previously demonstrated in this laboratory¹⁰ but not observed by others¹⁷. Additional studies will permit quantitative comparisons of the enzymatic activity of $p60^{src}$ of prokaryotic and eukaryotic origin.

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Propagation of foreign DNA in plants using cauliflower mosaic virus as vector

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Cauliflower mosaic virus (CaMV) is the best analysed member of the caulimoviruses, a group of small isometric plant viruses that contain a circular double-stranded genome (for review see ref. 1). As most plant viruses have an RNA rather than a DNA genome, caulimoviruses have attracted considerable interest due to their potential use as genetic vectors for plants. DNA purified from CaMV particles can infect plants and cause virus production when rubbed over the surface of susceptible leaves². Moreover, the entire chromosome of several CaMV strains can be propagated through bacterial hosts by plasmid and phage λ vectors and has been used successfully to infect plants^{3,4}. Recently, Howell *et al.*⁵ reported the insertion of an 8-base pair (bp) *EcoRI* linker molecule into the large 'intergenic' region of cloned CaMV strain CM 4-184 DNA without impairment of infectivity. Here we report the successful propagation of foreign DNA in plants using cauliflower mosaic virus as vector. We find that the size of foreign DNA that can be successfully propagated through virus particles has an upper limit of ~250 base pairs.

The nucleotide sequence of CaMV^{6,7} suggests that there are extensive regions of the genome which encode protein. To insert new DNA, however, it is necessary to identify non-coding or dispensable regions where insertions will not interfere with essential functions. To identify such regions, we selected a segment of DNA which specifies a phenotype in *Escherichia coli* that is easily detected and selected, and which is sufficiently small so as not to exceed a possible limitation on packaging capacity of the virion. This marker DNA was then inserted into the CaMV genome cloned in an *E. coli* plasmid vector.

The *E. coli* marker chosen for these trial insertions was a 65-bp fragment derived from the *lac* promoter-operator region⁸. In a multicopy plasmid, this *lac* operator fragment leads to a *lac* constitutive phenotype in a suitable host due to titration of *lac* repressor⁹. We provided the fragment with an asymmetrically positioned *EcoRI* restriction site and added synthetic *XhoI* linker molecules to its ends¹⁰. *XhoI* linkers were chosen because the position of a naturally occurring *XhoI* site on the chromosome of CaMV strain 1841 is covered by a deletion in the variant strain CM 4-184 (refs 11, 12), indicating a non-essential region of the genome. The *lac* operator with *XhoI* ends could be inserted easily to test this hypothesis. As an acceptor molecule we used DNA of plasmid pCaMV10, a derivative of

pBR322 carrying the complete genome of CaMV strain 1841 (refs 4, 12). The structure and orientation of two different *lac* operator inserts are given in Fig. 1.

To test whether a CaMV genome containing such inserts is infectious, the viral DNA was released from the plasmid vector by cleavage with *Sal*I restriction endonuclease and used to infect leaves of 'Just Right' turnip plants (*Brassica campestris* L.). About 200 ng of DNA per leaf were sufficient to cause 2–30 local lesions on mechanically inoculated leaves and led in all cases to symptoms typical of systemic infection¹³. The

appearance of symptoms was slightly delayed compared with infection by the parent molecules and the disease symptoms were milder. Viral DNA prepared from infected plants was analysed¹⁴. Figure 2a shows that the *lac* operator fragment can be recovered after *Xho*I digestion of the viral DNA. To determine whether only a fraction of the viral population contained the insert, four individual local lesions induced by DNA containing both types of inserts were transferred to several new plants. After systemic symptoms developed, viral DNA from each of the individual transfers was analysed. Figure 2b shows that the offspring of all eight independently transferred lesions carried the *lac* operator insert.

To determine whether the inserts were copied correctly during viral DNA replication we subcloned in plasmid pBR322 a *Hind*III restriction fragment containing the *lac* operator inserts at the *Xho*I site (Fig. 1) from either passaged CaMV DNA or from the parent plasmid used to infect the plants. DNA of these subclones was sequenced according to the Maxam–Gilbert procedure¹⁵. Figure 3 shows an example of the sequence comparison. No changes in the sequence of the two *lac* operator regions were detected. Regions adjacent to the site of insertion showed no sequence alteration compared with the pCaMV10 parent DNA sequence.

The stability of the inserts was checked after three successive transfers of virus derived from systemically infected tissue. All eight independent insert lines still contained the *lac* operator fragment. Some revertants occurred in the CaMV-EOP1 progeny, as indicated by the appearance of additional minor bands in the *Eco*RI restriction pattern. With CaMV-EOP2 (opposite orientation) no minor bands due to revertants could be detected. However, after five successive transfers and extended growth of the plants (2 months) the *lac* insert in both orientations was lost. In these conditions rare revertants seem to have a selective advantage over the insertion mutants which have slightly delayed growth.

To determine the maximum insertion capacity at the *Xho*I site, we inserted fragments of 256, 531 and 1,200 bp, respectively. The 256- and 531-bp fragments were derived from phage λ DNA cleaved with *Sal*I and *Xho*I (sites 44, 45 and 46 of a 1981 λ map update, F. R. Blattner *et al.*, personal communication). The 1,200-bp fragment, a *Sal*I segment of pUC5 containing the kanamycin resistance gene of Tn903 (Vieria and J. Messing, unpublished results), carries *Pst*I sites adjacent to its *Sal*I ends, with the kanamycin resistance gene inserted between the *Pst*I sites by G·C homopolymer tails. Infectivity assays using CaMV DNA containing these inserts were performed as described above. After development of systemic symptoms, viral DNA was prepared from infected leaves and analysed. Figure 4 shows the *Eco*RI digestion pattern of CaMV DNA derived from the 256- and 531-bp insertions; only the former was propagated stably. Insertion of 531 bp gave rise to three different deletion mutants in the region of insertion. These infections were apparent in plants only after a prolonged period: 5–6 weeks after inoculation as opposed to 2 weeks normally required for systemic development. In addition, attempts to recover infectious CaMV DNA molecules containing the 1,200-bp fragment were unsuccessful. Analysis of the viral DNA from plants that developed symptoms showed that the inserted fragment was lost, perhaps by recombination in the homopolymer tails as most of the isolates gained at least one additional *Pst*I site at the insertion point.

Thus, we have provided evidence for a non-essential region in the CaMV genome. Insertion of foreign DNA from two different sources into an *Xho*I restriction site within this region does not interfere with the infectivity of the viral DNA nor with virus production. However, the stability of the 256-bp λ DNA insert in a systematic survey has not been compared with that of the *lac* EOP inserts. The instability of the 64/65-bp *lac* promoter–operator inserts could be due to some peculiarity of the DNA sequence itself. Note for example the similarity of the *lac* UV5 Pribnow box to the 'TATAA' initiation signal sequence for RNA polymerase II in eukaryotes¹⁶. Whether the

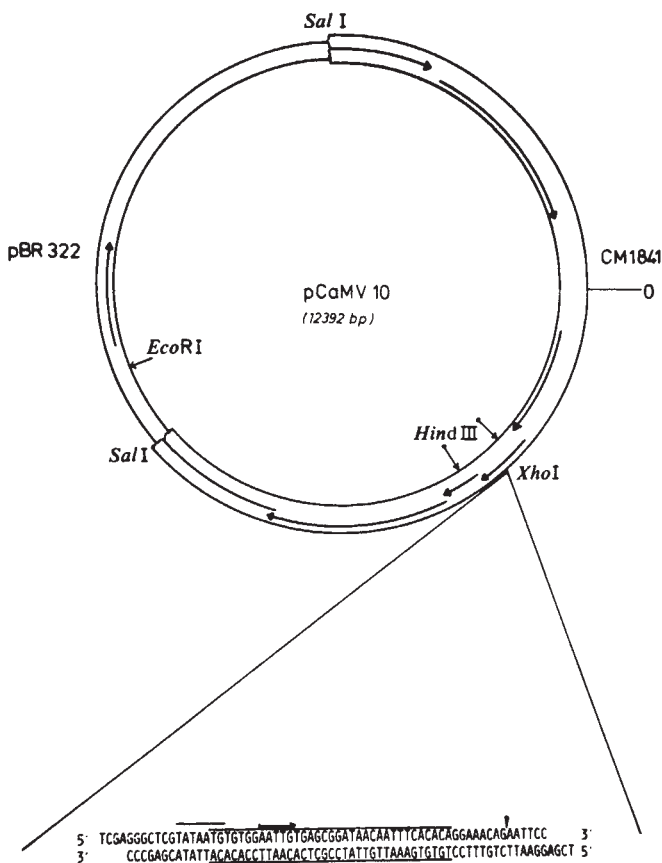
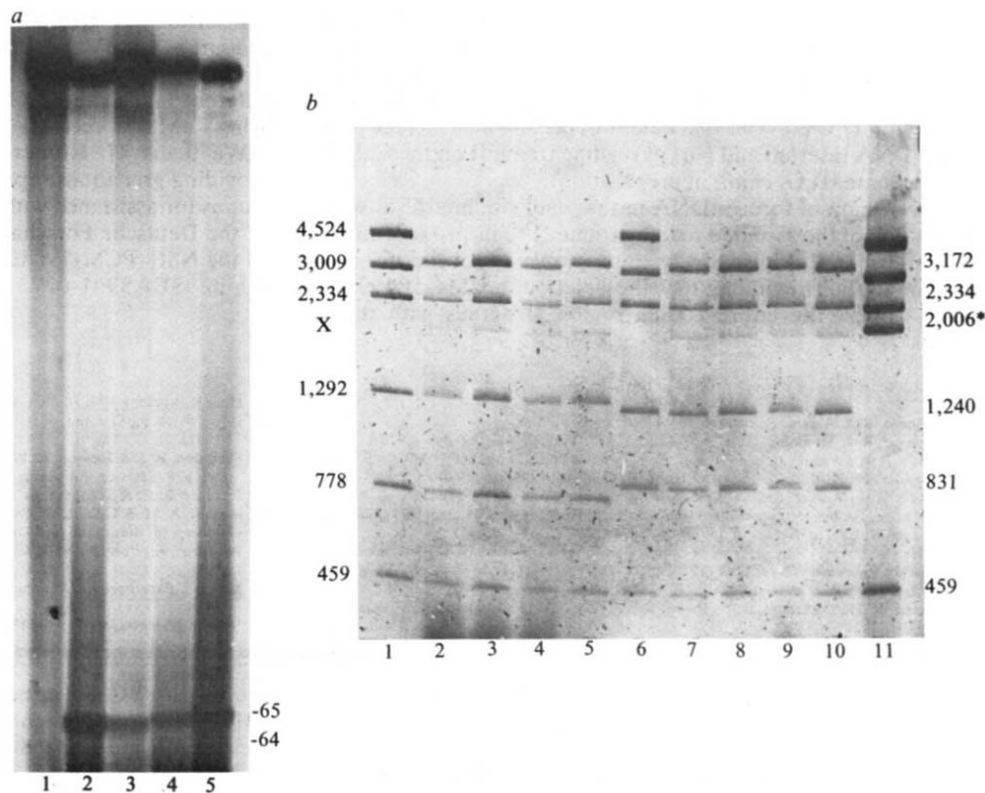


Fig. 1 Structure and orientation of the 1841-EOP2 *lac* operator insert at the unique *Xho*I site of the CaMV 1841 hybrid plasmid pCaMV10. A 55-bp *Hpa*II–*Eco*RI restriction fragment from the *lac* UV5 promoter–operator region of *E. coli* in plasmid pUR108-1 (ref. 8 and U. Rüther, unpublished results) was given flush ends by incubation with *E. coli* Pol I Klenow fragment (Boehringer) in the presence of dATP and dTTP¹⁷. After purification by electrophoresis through an 8% polyacrylamide gel, 1 pmol of fragment was ligated to 50 pmol of *Xho*I linker molecules (Collaborative Research)¹⁰. This step restores the *Eco*RI restriction site at the promoter distal end of the fragment. *Xho*I restriction endonuclease digestion (restriction enzymes from New England Biolabs, Boehringer or BRL) was used to cleave off superfluous linkers and a second 8% polyacrylamide gel was used to prepare the now *Xho*I-ended fragment. The purified fragment was ligated to pCaMV10 DNA linearized by *Xho*I. Hybrid molecules were identified after transformation of *E. coli* CSH 51 (ref. 18) and selection of ampicillin resistant bacteria on plates containing ampicillin (50 μ g ml⁻¹) and 5-bromo-4-chloro-indolyl- β -D-galactoside (40 μ g ml⁻¹). *lac* constitutive bacteria produce blue colonies in these conditions¹⁸. Two different isolates were analysed further; these carry the *lac* operator in both orientations in the CaMV genome. In addition, pCaMV10-EOP1 has lost one terminal nucleotide pair at the *Hpa*II end of the *lac* operator. The six open reading frames of CaMV are indicated by arrows, starting in a clockwise direction from the zero point of the map. The orientation of pBR322 relative to CaMV DNA is indicated by its unique *Eco*RI site and the reading frame of the β -lactamase gene. The *lac* operator insert of pCaMV10-EOP2 is enlarged at the bottom. The Pribnow box homology and the operator sequence are underlined. The arrow pointing to the right indicates the transcription start point in *E. coli*. The additional *Eco*RI site is indicated by ∇ . pCaMV10-EOP1 carries the *lac* operator in the opposite orientation and lacks one GC pair at the former *Hpa*II end of the fragment.

Fig. 2 *a*, Viral DNA was prepared from plants that had been infected with *SalI*-cut DNA of pCaMV10 (lane 1), pCaMV10-EOP1 (lane 3) and pCaMV10-EOP2 (lane 4). After cleavage by *XhoI* restriction endonuclease and labelling with [α - 32 P]dATP by polymerase I (Boehringer), the DNA was electrophoresed on a 10% polyacrylamide gel. Plasmid DNA of pCaMV10-EOP1 (lane 2) and pCaMV10-EOP2 (lane 5) was treated in the same way. The 64-bp operator fragment of -EOP1 can be distinguished from the 65-bp fragment of -EOP2, as indicated at the right-hand side. *b*, *EcoRI* restriction pattern of viral DNA derived from eight independent local lesion isolates of CaMV-1841-EOP1 and EOP2 that had been transferred to a set of new plants. Lanes 1, 6 and 11 contain plasmid DNA from pCaMV10-EOP1, pCaMV10-EOP2 and pCaMV10, respectively. Lanes 2-5 contain DNA from four different isolates of CaMV1841-EOP1; lanes 7-10, DNA of four independent isolates of CaMV1841-EOP2. The *EcoRI* fragment that has been modified by the insertions is indicated by *. The size of the fragments is given in base pairs. The introduction of the *lac* operator insert into the 2,006-bp *EcoRI* fragment also introduces an additional *EcoRI* restriction site and gives rise to two new fragments, the sizes of which (1,292 and 778 bp as opposed to 1,240 and 831 bp) vary depending on the orientation of the insert. X indicates a submolar fragment present in all *EcoRI* digests of CaMV DNA⁷. The 4,524- and 3,009-bp fragments are 'hybrid' fragments containing CaMV and pBR322 DNA, their CaMV moiety is part of the 3,172- and 2,334-bp fragments of the viral DNA.



transcription patterns of CaMV 1841-EOP1 or 1841-EOP2 differ from that of CaMV 1841 has not been determined.

A packaging limitation of CaMV particles for additional DNA seems to restrict larger inserts—an additional 531 bp could not be successfully propagated in similar experiments. Thus there seems to be an upper limit for additional DNA of 256–531 bp. Recombinant molecules having large insertions in the CaMV genome may replicate, as a whole, only in those cells which become initially infected after inoculation. Perhaps encapsidation occurs only after sufficiently small deletions have arisen and may be a prerequisite for cell-to-cell movement and

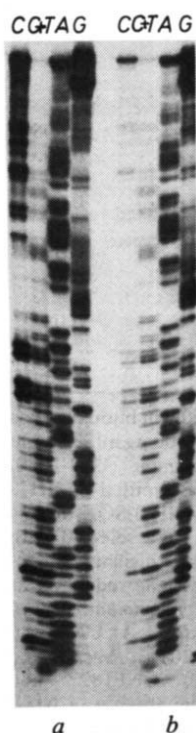


Fig. 3 Sequence comparison of the *lac* operator insert before and after passage through the plants. The respective *HindIII* restriction fragments (see Fig. 1) were subcloned in pBR322 (ref. 19). DNA of the hybrid plasmids was cleaved by *EcoRI*, labelled either with [γ - 32 P]ATP (Amersham) and T4 polynucleotide kinase (Boehringer) or by Pol I Klenow fragment plus [α - 32 P]dATP to label the opposite strand. After cleavage with *HindIII* and preparation of the fragments on 6% polyacrylamide gels, the DNA sequence of the inserts was determined as described elsewhere¹⁵. The figure shows an 8% sequencing gel of CaMV-EOP1 DNA (after passage) labelled with [α - 32 P]dATP (*a*) and pCaMV10-EOP1 DNA before passage through the plants (*b*). The readable sequence starts with 3' AACAAAT 5' in the middle of the *lac* operator (see Fig. 1, upper strand).

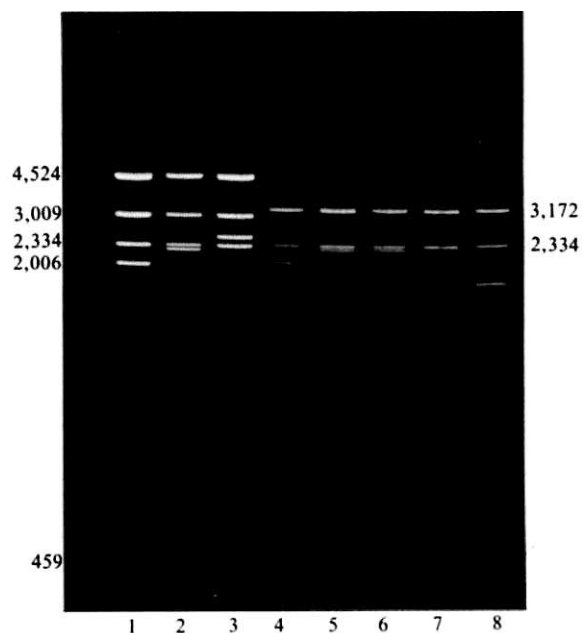


Fig. 4 *EcoRI* restriction pattern of λ DNA insertions in CaMV1841. Lane 1, *EcoRI*-cleaved plasmid DNA of pCaMV10; lanes 2 and 3, pCaMV10 containing a 256- and a 531-bp insert of λ DNA, respectively. The insertions at the *XhoI* site cause differences in mobility of the former 2,006-bp fragment. Lanes 4–8 contain viral DNA isolated from individual plants that showed symptoms after inoculation with the three plasmid DNAs cut by *SalI*. Lane 4 shows the wild-type CaMV1841 *EcoRI* restriction pattern. The mobility of the *EcoRI* fragment that contains the 256-bp insert seems identical to that of the parent plasmid (lanes 5 and 6). In contrast, the 531-bp insert produced three different deletions, each of which rendered the fragment carrying the insert smaller than the wild-type 2,006-bp fragment (lanes 7 and 8). Only the largest of these new fragments (lane 7) still contains sequences that hybridize to λ DNA (data not shown).

systemic development in the plant. Further experiments with a 363-bp insertion at the *Xho*I site of pCaMV10 support this assumption as there is an 'eclipse period' of >2 months before the plants start to develop symptoms of virus infection. Analysis of the viral DNA reveals that deletions occur which include part of the DNA inserted and part of reading frame II on the CaMV chromosome (B.G. *et al.*, in preparation).

As insertion of foreign DNA in the *Xho*I site interferes with expression of the assumed reading frame II^{3,4}, its product might be non-essential. Alternatively, this region may not code for any protein at all. The finding that the deletion in CM4-184 eliminates almost the entire coding region II agrees with these results¹².

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Insertion of bacterial DNA has been useful in probing the genome of CaMV. We are now investigating whether there are other regions suitable for insertions and whether inserts of different kinds can be used to express new proteins in plants using CaMV as vector.

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Role of the *nifA* gene product in the regulation of *nif* expression in *Klebsiella pneumoniae*

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The nitrogen fixation (*nif*) gene cluster of *Klebsiella pneumoniae* is linked to *his*, consists of at least 17 genes arranged in seven or eight operons^{1–4} (Fig. 1), and its expression is inhibited by oxygen (O₂) and fixed nitrogen (N) including ammonia, nitrate and amino acids (see ref. 5 for review). Previous studies have suggested that the *nifA* and *nifL* gene products respectively activate^{6–8} and repress^{9,10} *nif* expression. There has also been evidence, from mutants that express *nif* constitutively in the presence of fixed N, that regulation by fixed N involves the *gln* (glutamine synthetase) genes^{11,12}, and that their products act at the promoter of the *nifLA* operon^{13,14}. We have recently shown by direct measurement of transcription *in vivo* that the *nifA* and *nifL* gene products are, respectively, an activator and repressor of *nif* transcription initiation from all *nif* promoters except that of the *nifLA* operon¹⁵. We now report experiments aimed at clarifying the role of the *nifA* product and the interaction of the *nifLA* operon and *gln* genes in controlling *nif* expression. Having constructed plasmids that constitutively express the *nifA* gene and investigated *nif* expression in a variety of *K. pneumoniae* strains transformed by such plasmids, we show that, in the absence of *nifL* protein, *nifA* expression is sufficient to activate *nif* expression, even in the presence of fixed N and O₂. *gln*-mediated regulation of *nif* by fixed N exerts its effect by controlling *nifLA* expression. We have also confirmed that the *nifA* protein is temperature sensitive, thus explaining the absence of *nif* expression at 37 °C. Our results provide a strategy by which the constitutive expression of *nif* in the presence of fixed N and O₂ is stabilized and suggest a model for *nif* transcriptional regulation.

To investigate the regulation of *nif* expression by fixed N and O₂ and to clarify the role of the *nifA* product, we have used a variety of *K. pneumoniae* strains transformed with plasmids permitting constitutive expression of the *nifA* gene. The gene was cloned as a *Sal*I fragment (which also contained part of the 3' end of the *nifL* gene) in the multicopy vectors pACYC184 and pACYC177 (ref. 16). The fragment was inserted into the *Sal*I

site of pACYC184 in both orientations, giving pMC71A and pMC71B; in pMC71A *nifA* was expressed from the promoter of the tetracycline resistance gene (Tc^r). Insertion into the *Xho*I site of pACYC177 in both orientations gave pMC73A and pMC73B; expression of *nifA* in pMC73A was from the promoter of the kanamycin resistance gene (Km^r).

Table 1 Nitrogenase activities of *K. pneumoniae* strains with and without *nifA* plasmids

Strain	Nitrogenase activity as % of that in derepressed UNF928	
	–NH ₄ ⁺	+NH ₄ ⁺
UNF928 (<i>nif</i> wild-type strain)	100	0.005
UNF714 (<i>nifA</i>)	0	0
714 (pMC71A)	97	15
714 (pMC71B)	7	0.1
714 (pMC73A)	230	27
714 (pMC73B)	0.1	0.1
UNF1780 (<i>glnA100</i> → Nif [–])	0.05	0.075
1780 (pMC71A)	328	88
1780 (pMC71B)	257	1
1780 (pMC73A)	174	80
1780 (pMC73B)	0.005	0.005
UNF1827 (<i>glnA201</i> → Nif [–])	6	0.005
1827 (pMC71A)	276	86
1827 (pMC71B)	122	0.07
1827 (pMC73A)	279	70
1827 (pMC73B)	0.2	0
UNF1829 (<i>glnG36</i> → Nif [–])	0.1	0
1829 (pMC71A)	284	22
1829 (pMC71B)	226	0.03
1829 (pMC73A)	125	22
1829 (pMC73B)	0.06	0.07

Overnight cultures (4 ml) were grown anaerobically in bijoux bottles in NFDM²² containing 25 µg ml^{–1} histidine and 100 µg ml^{–1} serine (UNF928, UNF714 strains) or 100 µg ml^{–1} glutamine (UNF1780, UNF1827, UNF1829 strains) (–NH₄⁺). Serine was omitted in NH₄⁺-grown cultures (+NH₄⁺) which contained 10 mM (NH₄)₂SO₄. Strains carrying pMC71A and B and pMC73A and B were selected with 25 µg ml^{–1} chloramphenicol and 200 µg ml^{–1} carbenicillin, respectively. Nitrogenase activity was measured by acetylene reduction as described in ref. 22. Relevant genotypes are: *K. pneumoniae* strains, UNF928 (*hisD hsdR recA*), UNF714 (*hisD nifA hsdR recA*), UNF1780 (*hisD glnA100 hsdR recA*), UNF1827 (*glnA201 hsdR recA*) and UNF1829 (*glnG36 hisD hsdR recA*). UNF1780, UNF1827 and UNF1829 do not express *nifA* or *nifL*, even in the absence of NH₄⁺, because they lack the necessary *gln*-mediated positive control.

Table 2 β -galactosidase expression from the *nifH* promoter in strains with and without *nifA* plasmids

Strain	β -galactosidase units			
	$-\text{NH}_4^+$		$+\text{NH}_4^+$	
1. UNF921	3		3	
2. UNF921 (pMC71A)	1,953		2,267	
3. UNF921 (pMC71B)	716		125	
4. UNF931 (pJB31)	2,200		2	
5. MC1061 (pJB31)	3		2	
6. MC1061 (pJB31) (pMC71A)	6,027		4,246	
	$-\text{NH}_4^+$		$+\text{NH}_4^+$	
	$+\text{O}_2$	$-\text{O}_2$	$+\text{O}_2$	$-\text{O}_2$
7. UNF921	3	6	2	3
8. UNF921 (pMC71A)	2,250	2,160	1,570	3,000
9. UNF921 (pRD1)	22	2,200	8	6

Overnight cultures (4 ml) of strains 1–6 were grown anaerobically in bijoux bottles in NFDM²² containing $25 \mu\text{g ml}^{-1}$ histidine except for UNF921 (pRD1), $100 \mu\text{g ml}^{-1}$ serine ($-\text{NH}_4^+$) or $10 \text{ mM } (\text{NH}_4)_2\text{SO}_4$ instead of serine ($+\text{NH}_4^+$). Overnight cultures (10 ml) of strains 7–9 were grown in the same two media under a constant stream of air ($+\text{O}_2$) or $\text{N}_2(-\text{O}_2)$ in 100-ml conical flasks which were vigorously shaken. All cultures were grown at 28°C . β -galactosidase activities were measured as described in ref. 8. Relevant genotypes are: *K. pneumoniae* strains, UNF921 (*lacZ::nifH* ∇ (*his-nifH*)*recA hsdR*), UNF931 (*hisD lac recA hsdR*) and *E. coli*, MC1061 (*hsdR* Δ (*lacIPOZY*)X74). Plasmids pMC71A and B carry Cm^r and *nifA* genes, pJB31 carries Ap^r and *lacZ::nifH* genes and pRD1 is a conjugative plasmid carrying Ap^r Tc^r Km^r *his* and *nif* genes. Details of pJB31 construction will be published elsewhere.

Expression of *nifA* from these plasmids was checked in a *NifA*[−] strain UNF714. The results in Table 1 show that pMC71A and pMC73A both complemented the *nifA* mutation in UNF714. The low level of activity obtained with pMC71B was probably due to expression of *nifA* from an unidentified promoter on pACYC184, because a significant level was not obtained with pMC73B, indicating that no transcription initiation occurs between *nifL* and *nifA*.

Table 1 lists the nitrogenase activities of several different *K. pneumoniae* strains with and without the *nifA* plasmids and in the presence and absence of NH_4^+ . Nitrogenase activity in the *nif* wild-type strain UNF928 is repressed in the presence of $10 \text{ mM } \text{NH}_4^+$. However, strain UNF714, when restored to *Nif*⁺ by pMC71A or pMC73A, shows 15–30% constitutive nitrogenase activity (activity in the presence of NH_4^+ as a percentage of that in the absence of NH_4^+). Constitutive *nif* expression in the presence of NH_4^+ was also examined by measuring β -galactosidase activity in a strain (UNF921) with a *nifH::lacZ* fusion (Table 2). In this strain the *lacZ* gene is fused to the *nifH* promoter and *his-nifH* is deleted (M. Merrick, unpublished). Strain UNF921 carrying pMC71A shows 100% constitutive β -galactosidase expression from the *nifH* promoter. The difference in degree of constitutive expression at the transcriptional (β -galactosidase activity) and nitrogenase activity levels may be due to a physiological effect on nitrogenase activity in an NH_4^+ -grown culture. The effect of O_2 on *nif* expression could be examined in UNF921 (pMC71A); nitrogenase activity could not be used because the enzyme is inactivated by O_2 . The results (Table 2) show that β -galactosidase expression was 70–100% constitutive in UNF921 (pMC71A) grown in the presence of O_2 with and without NH_4^+ .

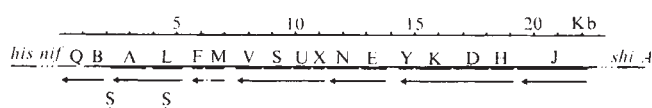


Fig. 1 Map of *K. pneumoniae* *nif* genes^{1–4}. The arrows indicate *nif* operons and their directions of transcription. The *SalI* restriction fragment used in the construction of *nifA* clones is denoted by S–S. Kb, kilobases.

Thus these results show that *nifA* protein is active in the presence of fixed N and O_2 , and that in the absence of *nifL* protein only the *nifLA* operon is regulated by fixed N or O_2 .

To examine the possibility that the *nifA* protein is the only *nif* gene product required for transcriptional activation of the *nifH* promoter, we constructed a small, multicopy plasmid, pJB31, carrying a translational *nifH::lacZ* fusion. *nifH* and *lacZ* were fused by *Bal*31 digestion from codons 147 and 8 of the respective genes followed by blunt-end ligation and selection of clones which had β -galactosidase activity. The results in Table 2 show that *lac* was not expressed from pJB31 in an *Escherichia coli* strain, MC1061, while in the *Nif*⁺ strain UNF931 *lac* expression from pJB31 was under normal *nif* regulation. When pMC71A was also present in MC1061, *lac* was expressed at a high level from pJB31 even in the presence of NH_4^+ . Thus, the only *nif* gene product required for activation of the *nifH* promoter is that of *nifA*. SDS-polyacrylamide gel electrophoresis of pulse-labelled extracts from UNF921 (in which *his-nifH* is deleted) and UNF921 (pMC71A) also indicated that the *nifA* protein was the only *nif* gene product required for expression from the *nifJ* promoter.

The constitutive expression of *nif* in UNF714 carrying pMC71A or pMC73A suggested that *gln*-mediated regulation of *nif* expression acted only at the promoter of the *nifLA* operon. To test this hypothesis we investigated the effect of the *nifA* plasmids on *nif* expression in three *gln* mutants, which normally do not express *nif*, presumably because a *gln* protein regulates transcription of the *nifLA* operon. The results (Table 1) show that the expression of *nif* was independent of *gln* when plasmids that constitutively expressed *nifA* were present in these mutants, indicating that only the *nifLA* promoter was directly regulated by the *gln* system.

The plasmid pMC71B restored higher nitrogenase activity to the *Gln*[−] strains than to UNF714 (Table 1), perhaps due to the absence of *nifL* expression in the *gln* mutants. We have found that the *nifL* protein repressed *nif* transcription¹⁵ and it is possible that in its absence less *nifA* protein is required to give a high level of *nif* expression. A result consistent with this observation is that a *nifL* mutant derepressed at a faster rate and gave

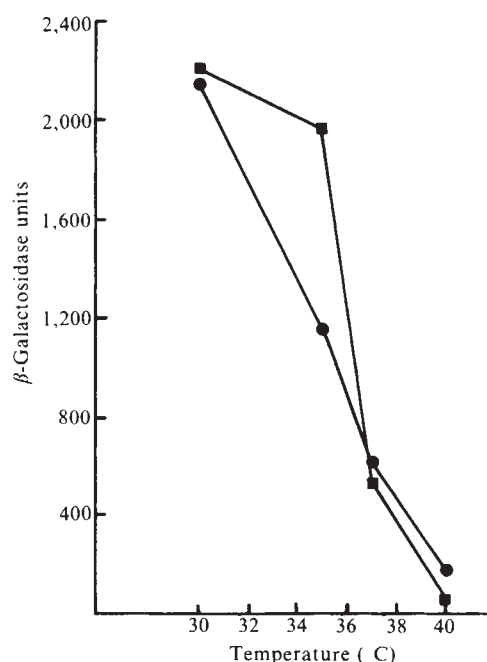


Fig. 2 Effect of temperature on *nif* expression in UNF921 (pMC71A) (●) and UNF921 (pRD1) (■). Cultures (10 ml) in 25-ml conical flasks were grown anaerobically in NFDM containing serine ($100 \mu\text{g ml}^{-1}$)²². UNF921 (pMC71A) cultures also contained histidine ($25 \mu\text{g ml}^{-1}$) and chloramphenicol ($25 \mu\text{g ml}^{-1}$). After overnight growth at four different temperatures β -galactosidase activities were measured as previously described⁸.

a higher level of nitrogenase activity than the wild-type (ref. 17 and M.C.C., S. Hill, E. Kavanagh and F.C.C., in preparation). The repression of *nif* by NH_4^+ in the *gln* mutants carrying pMC71B is probably due to a blocking of *nifA* expression by vigorous transcription from the Tc^r promoter in the opposite direction. We have found that transcription from the Tc^r promoter blocked transcription from the opposite direction in a similar promoter arrangement¹⁵.

Nitrogenase from *K. pneumoniae* is active at 37 °C but *nif* is not expressed at this temperature¹⁸. There is evidence that this temperature sensitivity is mediated by the *nifL* and/or *A* gene products^{17,19}. We therefore examined the effect of temperature on the activity of *nifA* protein by comparing β -galactosidase expression in the *nifH::lacZ* fusion strains UNF921 (pMC71A) and UNF921 (pRD1) at different temperatures. Strains carrying pACYC184 grow in the presence of Tc at the temperatures used and this shows that transcription initiation from the Tc promoter on pMC71A is not temperature sensitive. Figure 2 shows that β -galactosidase activity decreased in both strains with an increase in temperature. As *nifA* protein is constitutively synthesized from the Tc^r promoter in UNF921 (pMC71A) and no *nifL* protein is present, this result shows that the *nifA* protein is temperature sensitive. A similar result was obtained with a plasmid on which *nifA* alone was expressed from the *nifLA* promoter, which is known not to be temperature sensitive^{17,19}. The *nifA* protein has some activity at 37 °C and the observation that *nifL* mutants express *nif* at 37 °C¹⁷ is probably due to a lower requirement for *nifA* protein in the absence of a functional *nifL* protein (see above).

Our results show that *nif* expression can be switched on constitutively by inactivating *nifL* and fusing *nifA* to a constitutive promoter. These and other recent results provide strong evidence for the following model of *nif* transcriptional regulation. In *nif* derepressing conditions a *gln* protein activates transcription of the *nifLA* operon. The *nifA* protein then activates transcription of the other *nif* operons while the *nifL* protein is maintained in an inactive form by interaction with a regulatory metabolite. A probable candidate for this role is the purine nucleotide ppGpp which has an important regulatory role in conditions of N starvation^{20,21}. When fixed N or O_2 is added to a derepressed culture the level of the regulatory metabolite is decreased and the *nifL* protein becomes a repressor of transcription at the promoters of all the *nif* operons except that of *nifLA*. A rapid decrease in the level of ppGpp in these conditions would lead to the transition of RNA polymerase to a different structural form²¹ which would probably be less efficient at initiating transcription from *nif* promoters. Therefore, the repressor activity of *nifL* protein and the altered RNA polymerase would both contribute to a rapid *nif* switch-off on addition of fixed N or O_2 . In the presence of fixed N a *gln* protein represses transcription from the *nifLA* promoter, thus concomitantly removing the *nif* repressor and activator. Repression of *nif* in cultures grown in N-rich media would therefore be maintained solely by the absence of the *nifA* protein.

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cDNA sequences of human glucose 6-phosphate dehydrogenase cloned in pBR322

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Glucose 6-phosphate dehydrogenase (G6PD) catalyses the first reaction of the so-called oxidative pathway of glucose metabolism¹. In many cells, in addition to providing pentose sugars required for nucleic acid synthesis, G6PD has a major role in providing an adequate supply of reducing power in the form of NADPH (ref. 2). The enzyme has been purified and characterized from many sources³. G6PD from mammalian cells consists of a homodimer or a homotetramer, with a subunit molecular weight of ~58,000 (ref. 4), and its structural gene is located on the X chromosome⁵. Genetic variation of G6PD is extensive in humans. Many variants associated with relative deficiency of enzyme activity ('G6PD(-)') are polymorphic in various populations and have probably been selected for by *Plasmodium falciparum* malaria⁶. A number of G6PD(-) variants can cause either acute or chronic haemolytic anaemia and therefore have considerable clinical importance⁷. We considered that a knowledge of the G6PD gene would be of interest because it may be representative of a large number of genes and could help to elucidate in detail the basis for various forms of G6PD deficiency. Here we report how G6PD cDNA sequences have been cloned starting from human mRNA in which G6PD mRNA is present at an abundance of less than 10⁻⁴.

Human diploid fibroblasts were grown from a primary skin explant by standard techniques⁸. Approximately 10⁹ cells were used to prepare total RNA by the guanidine HCl method⁹. Enrichment for G6PD-specific mRNA was obtained by successive steps, consisting of oligo(dT)-cellulose chromatography and two successive sucrose gradient centrifugation runs, as will be described in detail elsewhere (D.T., M.G.P., G. Battistuzzi and L.L., in preparation). The purification was followed by a specific assay based on cell-free translation (see Fig. 1). From the enriched mRNA (Fig. 1, tracks 4, 7), cDNA was prepared using oligo(dT)-primed reverse transcriptase according to Sobel *et al.*¹⁰, except that the concentration of oligo(dT) was 5 $\mu\text{g ml}^{-1}$ and that of each deoxynucleoside triphosphate was 1 mM. From this cDNA, double-stranded DNA was obtained using *Escherichia coli* DNA polymerase¹¹, and after C-tailing¹² it was annealed to G-tailed plasmid pBR322 (ref. 13) and used to transform *E. coli* HB101 (ref. 14). After plating on tetracycline-agar plates, 4,750 colonies were observed, of which 3,470 were found to be ampicillin sensitive, indicating that they contained a plasmid with a double-stranded DNA insert. From an estimate of the abundance of G6PD mRNA in the reverse-transcribed preparation, we expected that 5–10 should contain G6PD sequences.

The 3,470 colonies were screened by two approaches. First, RNA from cultured HeLa cells was purified as outlined in Fig. 1

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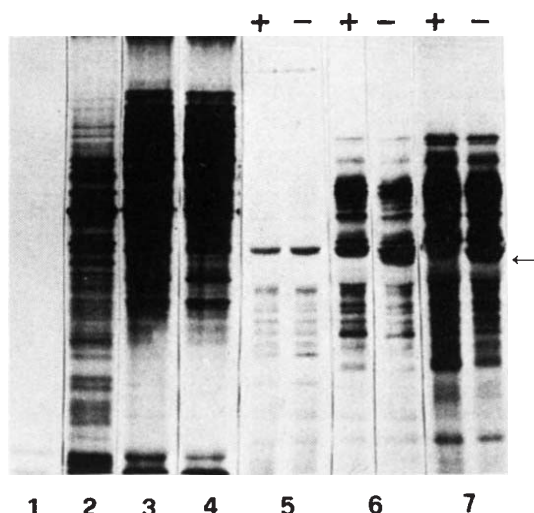


Fig. 1 SDS-polyacrylamide gel electrophoresis analysis of cell-free translation product of human fibroblast mRNA enriched in G6PD-specific mRNA. After incubation in a reticulocyte cell-free system, as described by Pelham and Jackson²⁰, samples were either mixed with an equal volume of twice concentrated 'sample' buffer²¹, or processed for immunoprecipitation as follows. Each 25- μ l sample was diluted with 25 μ l phosphate-buffered saline, containing 0.5% NP40, 100 μ g ml⁻¹ *p*-methylsulphonyl fluoride, 50 μ g ml⁻¹ TPCK, 20 μ g ml⁻¹ *N* α -*p*-tosyl-L-lysine (this solution will be referred to as PBS-inhib). Each diluted sample was next split into two identical 25- μ l aliquots. To one was added 50 μ l of a 1:50 dilution (in PBS-inhib) of a rabbit anti-human G6PD antiserum (the activity of which was sufficient to immunoprecipitate 0.5 μ g of pure G6PD, as assessed by immunodiffusion in agar). To the other aliquot was added the same amount of antiserum, which had been previously incubated for 2 h at 20 °C followed by 15 h at 4 °C with 1 μ g of authentic G6PD purified from human erythrocytes²². After 1 h at 20 °C, to each tube 20 μ l of a 10% suspension of formalin-fixed *Staphylococcus aureus* in PBS-inhib was added²³. After incubation for 1 h at 4 °C with intermittent rotary shaking the mixture was centrifuged for 3 min at 1,200g and the sediment washed three times by centrifugation with PBS-inhib. The sediment was finally resuspended in 40 μ l of twice-concentrated sample buffer²¹, boiled for 2 min and re-centrifuged. The final supernatants from the above procedure and samples not subjected to immunoprecipitation were applied to a 10% polyacrylamide gel²¹. At the end of the run, the gel was fixed in 10% acetic acid and processed for fluorography²⁴. Lane 1, cell-free product with no RNA added; lanes 2-4, cell-free products obtained with various additions (2, total poly(A) containing RNA; 3, RNA from sucrose gradient fractions sedimenting between 18S and 21S; 4, RNA fractions with similar S value from a second gradient to which the pooled 18-21S RNA from the first sucrose gradient had been applied). Lanes 5-7 are like 2-4, but cell-free translation was followed by immunoprecipitation as described above. In each pair + and - indicate addition and omission, respectively, of cold G6PD. An arrow indicates the position of authentic G6PD.

legend and further fractionated by agarose gel electrophoresis²⁵. At the end of the run, the gel was sliced, and RNA eluted from individual slices and assayed for G6PD synthesis (as in Fig. 1). From the RNA from each of two gel slices enriched in G6PD mRNA, and from one adjacent slice devoid of G6PD synthetic activity, ³²P-labelled cDNA was prepared with reverse transcriptase (see ref. 10, with the modifications mentioned above). The cDNA obtained (having a specific radioactivity of $\approx 10^8$ c.p.m. per μ g) was used as probe in a colony hybridization assay¹⁵. Of 595 colonies positive with the G6PD mRNA probes, 132 were also positive with the G6PD mRNA-free probe. The remaining 463 colonies were regarded as including good candidates for containing G6PD-specific sequences. Second, we reasoned that, because of the relative paucity of G6PD mRNA in the material originally cloned, clones with 'abundant sequences' could be discarded. To this end, 23 out of the original 3,470 colonies were picked at random, and their nick-translated DNA inserts again used as probes in colony hybridization tests. Probes that picked up more than 10 colonies were taken to represent relatively abundant cDNA species, and enabled us to discard 71 of the 463 above colonies. A total of 140 additional abundant sequence clones were similarly discarded by using as probe cDNA copied from total poly(A)-containing HeLa cell RNA.

Bacterial cells from the remaining 252 colonies were grown in 36 groups of 7 and DNA was extracted and tested for G6PD-

specific sequences by a 'positive selection' assay (see Fig. 2). With three groups of plasmids we observed a band having the molecular weight of G6PD, which disappeared when authentic, pure, nonradioactive red cell G6PD was added to the cell-free translation product before addition of the antiserum (Fig. 2a). All colonies corresponding to the above three groups were individually grown up, and the respective DNA preparations were retested: four were positive (Fig. 2b).

The length of the G6PD-specific DNA sequences present as inserts in these four plasmids was determined by agarose gel electrophoresis after cleavage with *Pst*I endonuclease (Fig. 3). Insert GD6222 has two *Pst*I sites, and the three fragments are 480, 280 and 220 base pairs (bp) respectively. Insert GD5919 has one *Pst*I site, the two fragments being 395 and 230 bp respectively. Insert GD3918 is 370 bp long. Insert GD6405 has

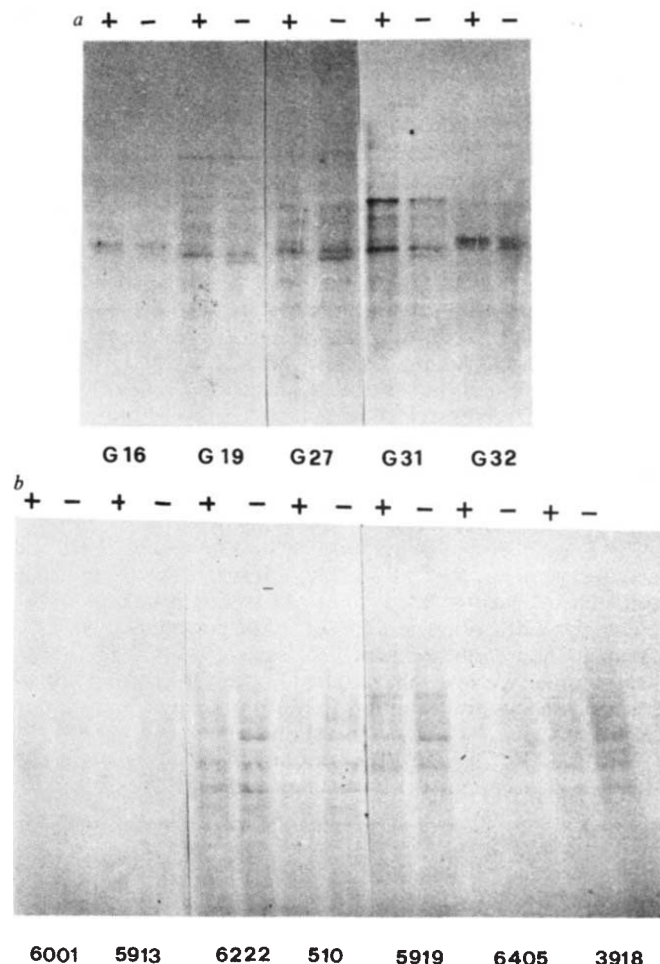


Fig. 2 Selection of G6PD-specific mRNA by hybridization with plasmid DNA. *E. coli* transformants, pooled in groups of seven (a), were grown overnight and plasmid amplification was obtained by the use of chloramphenicol²⁶. Plasmid DNA was extracted²⁶, purified by passage through a Bio-Gel A150 column²⁸ or by banding in *CsCl*²⁷, and treated with *Pst*I. Aliquots containing 5 μ g of DNA were spotted on nitrocellulose filters (Millipore HAWP 047), which were then placed for 3 min on each of three subsequent filter paper pads soaked, respectively, in: 0.5 M NaOH, 1.5 M NaCl; 1 M Tris-Cl pH 7.5, 2 $\times$ SSC; and 2 $\times$ SSC. Finally, the filters were baked in a vacuum oven for 2 h at 80 °C. HeLa cell RNA (5 μ g), purified through the first sucrose gradient step (see Fig. 1), was hybridized to the filters in 50% formamide, 750 mM NaCl, 100 mM Tris-Cl pH 7.5, 2 mM EDTA, 0.4% SDS, 0.6 μ g μ l⁻¹ tRNA for 20 h at 37 °C. After hybridization, the filters were washed 10 times at 60 °C in 150 mM NaCl, 10 mM Tris-Cl pH 7.5, 1 mM EDTA, 0.5% SDS, twice in the same buffer minus SDS, and finally boiled for 60 s in 300 μ l of H₂O in the presence of 10 μ g tRNA. The RNA was next extracted once with 150 μ l phenol (which had been previously saturated with 10 mM Tris-Cl pH 7.5), and 150 μ l of chloroform/isoamyl alcohol (24:1) and then precipitated with ethanol. Cell-free translation of this RNA, subsequent processing and the meaning of + and - symbols were as in Fig. 1. It is seen that groups G19, G27 and G31 have a band in the position of G6PD (arrow). b Shows results of similar experiments carried out with individual plasmids from the above positive groups. It is seen that nos 6222, 5919, 6405 and 3918 give a signal in the position of G6PD.

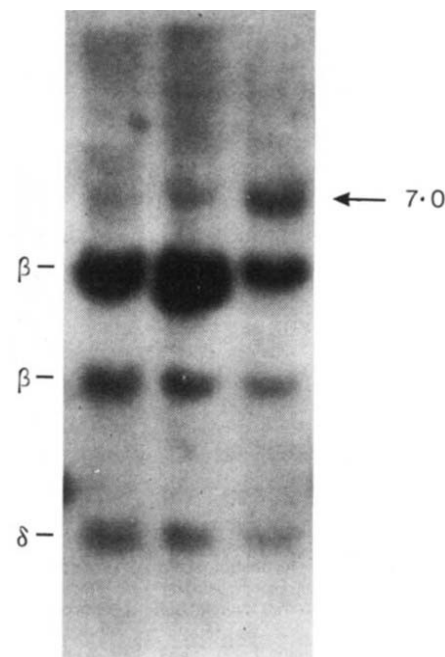
one *Pst*I site, the two fragments being 1,250 and 245 bp respectively. The length of the G6PD coding sequence, calculated from the subunit molecular weight, is about 1,650 nucleotides. In denaturing agarose gels, G6PD mRNA runs slightly behind 18S HeLa cell rRNA, and from this we estimate a size of about 1,900 nucleotides (D.T., M.G.P., G. Battistuzzi and L.L., in preparation). We have not determined what portion of the mature mRNA sequence is covered by the clones we have isolated.

The identification of the clones reported here rests on their capacity to hybridize with a specific mRNA. It was possible that the polypeptide band seen on SDS gels was not G6PD, but a different protein, perhaps another dehydrogenase, which cross-reacts with the anti-G6PD antibody used and which happens to have the same subunit size as G6PD. This was disproved by the finding that a digest of the radioactive band eluted from the gel gave a fingerprint pattern identical to that of authentic G6PD (D.T., M.G.P., G. Battistuzzi and L.L., in preparation). Matching nucleotide sequence with protein sequence is not possible in this case, because only fragmentary amino acid sequence data are available for G6PD. However, for two clones tested thus far, we have obtained direct evidence that they correspond to human X-chromosome sequences. Indeed, by Southern blot analysis¹⁶, ³²P-labelled pGD6405 yields a single band when hybridized to human DNA digested with endonuclease *Eco*RI. The signal is stronger with female (46, XX) than with male (46, XY) DNA, and even stronger with DNA from a (47, XXX) female (see Fig. 4). Another set of Southern blots for pGD3918 also yields a single band with *Eco*RI-digested human DNA. This band was not seen when DNA from Chinese hamster ovary (CHO) cells was similarly tested, but it was again seen with DNA from a human-hamster hybrid cell line, obtained in this laboratory, and characterized as expressing human G6PD and having a CHO karyotype plus the human X. Thus, these clones, identified as containing G6PD sequences by a translation assay, are found by an independent criterion to be X-linked.

The human globin genes have been cloned, mapped and partially sequenced^{17,18}. Recently, a human X-chromosome sequence has been isolated¹⁹, but its function is unknown. We believe those described here to be the first cloned sequences for a human 'household' gene which is also located on the X-chromosome. We hope they will be useful for investigating its structure and its transcription in various normal and mutant cells.

We thank R. Di Lauro for continuous encouragement and advice, M. Iaccarino, G. Battistuzzi and D. Schlessinger for

Fig. 4 Hybridization of G6PD-specific cDNA probe to human DNA. Plasmid pGD6405 was purified as described in Fig. 2 legend. A human β -globin probe, to be used as an internal control, consisted of a 4.4-kilobase (kb) *Pst*I genomic DNA fragment³⁰, comprising the entire β -globin gene. Human DNA was extracted from cultured fibroblasts³¹, digested with *Eco*RI endodeoxyribonuclease and electrophoresed on a 0.8% agarose gel. After transfer on to a nitrocellulose filter¹⁶, hybridization was carried out³² with nick-translated³³ pGD6405 and β -globin DNA. After washing and drying autoradiography was carried out (6 days exposure). Lane 1, DNA from a normal male; lane 2, DNA from a normal female; lane 3, DNA from a female with a 47, XXX karyotype. The bands labelled β on the left-hand side correspond, by comparison with suitable molecular weight markers, to the expected²⁸ 5.2- and 3.6-kb β -globin *Eco*RI DNA fragments. The band labelled δ corresponds to the expected²⁸ cross-hybridizing 2.25 kb 5' δ -globin *Eco*RI DNA fragment. All these bands were absent when the β -globin probe was omitted from the hybridization mixture. The band marked by an arrow on the right-hand side, which was absent when pGD6405 was omitted from the hybridization mixture, corresponds to 7.0 kb. It is seen that the β -globin signals are weaker with XXX DNA, presumably due to differences in DNA transfer efficiency. By contrast, the GD6405 signal increases progressively, with X-chromosome dosage, from left to right.



many helpful discussions, B. Paterson for useful suggestions, C. Salzano, M. Terracciano and G. De Simone for technical assistance, Professor A. De Flora for a gift of anti-G6PD antiserum, Dr L. Chasin for some CHO cell mutants, Dr S. Wolf and Professor B. Migeon for a sample of DNA from (47, XXX) cells, and Dr E. Avvedimento for the β -globin probe. Portions of this work benefitted from financial support given to our laboratory by the WHO/UNDP/World Bank Tropical Diseases Research Programme.

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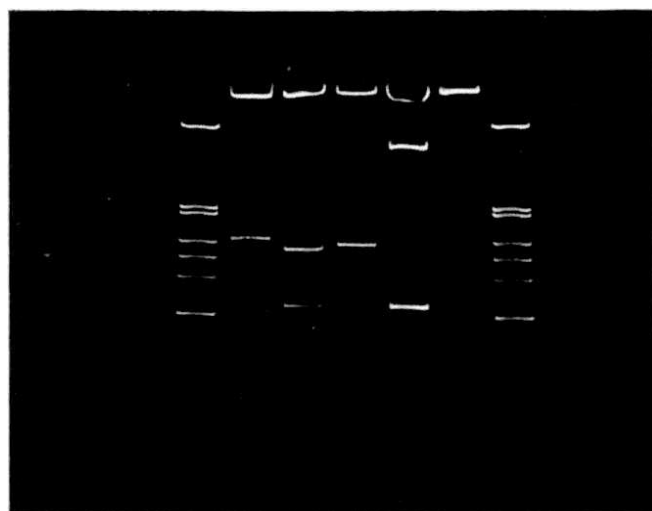


Fig. 3 Size of G6PD-specific cloned sequences, as determined by electrophoresis on a 5% acrylamide gel²⁹. The two lanes at the extreme right and at the extreme left show marker DNA fragments consisting of pBR322 digested with *Hinf*I. The remaining lanes show the patterns for plasmid DNA, purified and digested with *Pst*I endonuclease, from the following clones (see Fig. 2). Lane 1, 6222; lane 2, 5919; lane 3, 3918; lane 4, 6405; lane 5, pBR322.

BOOK REVIEWS

Darwin through a purple haze

A.J. Cain

THERE are already far too many books about Darwin, and most of them tell us more about their authors than their subject-matter. It would be an injustice to leave Mr Brent's book in that category without considerable qualification. It will certainly be widely read, and for many people it may be their first introduction to Charles Darwin's mental, moral and hormonal development. Mr Brent is a well-practised writer — he has already produced three novels and, according to the dust jacket, "ten pseudonymous thrillers, a study of Hindu gurus, two narrative histories, and several biographies". When he gets down to straightforward narrative and to psychological surmise, he is in his element; and he has certainly worked over a great deal of unpublished material relating to Charles's relations with his father, his sisters and other relatives, his early flames, his wife and his numerous friends. He has also profited, as he makes clear, from a number of other studies of Darwin and Darwinism; to that extent, the book is one of the best and most up-to-date accounts of Charles Darwin as a man that one can find.

Unfortunately, it is far from satisfactory in a most predictable way; the science in it is so light-weight that yet again we have Hamlet without the Prince, a sort of *Himmelfarb rediviva*. When will people of only literary training (and I include philosophers and some historians) realize that to write the history of a scientist they must intuit not only into his complexes and sex life but into his science as well?

The book is obviously aimed at the general market to be created by the centenary of Charles Darwin's death, already partly excited by the anti-Darwinian rubbish recently appearing in the popular press and on television and radio. To ensure a wide acceptance, the author has decorated it freely with purple passages which do not accord well with the generally excellent style in the narrative parts. Indeed, the first page after the Introduction, beginning "The boy is sturdy, high-complexioned, with fine, chestnut-coloured hair now in some disarray" and containing the remarkable sentence "Briefly, in delighted alarm, pigeons flick to and fro against the bulbous clouds" may even put off some who would find better things later.

Much of this could be disregarded, were not the same inflation constantly used on Charles Darwin himself. His attitude towards marriage was somewhat "monstrous" (p.249), his approach to his main task was "almost inhumanly thorough" (p.281), he was capable of "an

Charles Darwin: A Man of Enlarged Curiosity. By Peter Brent. Pp.536. ISBN UK 0-434-08595-2; ISBN US 0-16-014880-2. (Heinemann, London/Harper & Row, New York: 1981.) £12.50, \$20.75.

almost frightening objectivity" (p.320); many such expressions combine to build a picture of almost a maniac. They are reinforced by numerous suggestions of duplicity (p.187), hypocrisy (p.475), eagerness to duck criticism (p.229) and so on, which make the book a God-sent mine of reference for any fundamentalist wanting to denigrate Darwin. It is true that Mr Brent, having made these remarks often qualifies them; but as Paul Hazard reminds us, so-called refutations of Spinoza served to spread his ideas, and many of Mr Brent's self-rejected suggestions have now got into print. In much of what he judges so harshly, I see little more than very human vacillations, and not infrequently early Victorian modes of expression which he has taken rather too literally.

Mr Brent has done his best with Darwin's sex-life, in spite of a plentiful lack of material; indeed, at one point he speaks of the prurience of a biographer. His suggestions about Charles's relations with his sisters, and their over-mothering him are excellent, as is his perceptive account of his relations with his father. But when (p.263) he sees Emma as offering Charles "another aspect of the same archetype, Lady of the Manor and Peasant" he himself comments that "perhaps it gives it [this strand in their relationship] a false importance even to select it for comment" — and goes on immediately to emphasize it. And he just cannot resist a remark of one of Charles's female friends (p.61) regretting she was not there to meet him and "to make a *beast of myself* in the *strawberry beds*". Mr Brent gets quite excited about those strawberry beds, and his comment "One sees them [Fanny and Charles] at their languid orgy, half children, half adults" etc. etc. is worthy of *Cold Comfort Farm*; the beds recur again on p.140 ("the sprawling hours among the strawberry beds") and even again on p.501 ("the young Charles delirious with summer among the strawberry beds" etc.).

A great deal of what Mr Brent writes is well-written, sensitive, perceptive; it is a pity he should spoil the book, both in this way, and by setting up straw men to demolish, and by suggesting mysteries where they hardly exist in order to over-emphasize Charles's achievements. To

refer to Charles as "the hearty outdoor dullard of legend" makes one curious as to what reputable biographer ever suggested he was a dullard. To refer to Cuvier as enigmatic, or Lamarck as brilliant but enigmatic hardly does justice to their biographers, let alone the men themselves.

Equally, in the opposite direction, Charles Darwin did so much so well that he does not need to have ascribed to him ideas which he took from others. His vision of the Unity of Nature (p.289) was a commonplace to his grandfather, as were the ideas of trees as compound animals (p.317), sensitive plants as furthering the analogy between animals and plants (p.308) and the female bosom as an object of special interest (p.319), even, to Erasmus Darwin and others, the origin of the curve of beauty.

Except in dealing with the Galapagos Islands and Darwin's finches, which have been too well written-up to allow serious mistakes, Mr Brent hardly touches without error the actual science which Darwin so avidly investigated and extended. *Amphioxus* is tropical only if Plymouth is too. *Megatherium* appears on p.156 as "an enormous forerunner of the giant sloth" and on p.198 as "the prehistoric armadillo" — in fact it was one of the giant sloths and not a precursor of anything. The "genus *Brachipoda*" (p.403) is a hopeless misnomer, as is the "species *Canidae*" (p.472), and the cirripedes had not been molluscs since Lamarck (1818). Mr Brent has no patience with the mere details of natural history, like other authors I have reviewed recently, yet to Darwin they were the absolute basis of his science. It is not surprising that Mr Brent cannot really understand why Darwin laboured so long at his barnacles, and has to put it down to Darwin's character in the end.

It is clear from Mr Brent's references to sexual selection (p.480) that he has no more understood the present standing of that topic than Gertrude Himmelfarb understood the present standing of natural selection. To comment on sexual selection that the exercise of volition by a female

... muddled the simplicity of the original vision. It was an element which conflicted with the impersonal logic of a selection process that derived only from the interaction of organism and environment. . . .

is to show that what fascinates Mr Brent is ideas only (not their relevance to the actual world which is what fascinates a scientist). For him, natural selection "has become a staple of the everyday rhetoric through which we now define ourselves" (p.509) and it is his lack of knowledge of actual

work on natural selection that enables him to repeat the old cliché. What he calls the underlying logic of evolutionary theory "seems to say no more than that survivors survive". This hoary fallacy has been refuted many times, but he does not say so.

Mr Brent must surely have a considerable readership; his abilities in his own line are outstanding. The present book should be asked for as "the latest Peter Brent", rather than as a successful study of Darwin. No scientist need bother with it. □

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Rare air of motion

R. Hide

Dynamics of the Upper Atmosphere. By Susumo Kato. Pp.233. ISBN 90-277-1132-1. (Reidel: 1980.) Dfl. 57, \$29.95.

TECHNIQUES for investigating high-level atmospheric motions have advanced considerably over the past 20 years, with the introduction of rockets, satellites and high-powered radar on the observational side, and the advent of computers for the analysis of data and their theoretical interpretation in terms of basic physical processes.

Professor Sato's monograph — the first in a series, *Developments in Earth and Planetary Sciences*, stressing Japanese work but written in English — is a mathematically orientated introduction to the study of these motions and it reflects not only the author's main research interests but also (I imagine) his activities as a teacher of applied mathematics. The book deals in a straightforward and fairly standard way with the basic equations of dynamics, thermodynamics and electrodynamics applied to the atmosphere. These equations are used to describe the main properties of acoustic gravity waves and atmospheric tides and their manifestation in observations of the ionosphere. The presentation is strongly slanted towards those who are primarily interested in mathematical aspects of atmospheric wave propagation but many observational data are given in this profusely illustrated book, with its 106 diagrams and many tables.

Most atmospheric physicists would find something useful in the book, but it is possibly too narrow and, in parts, too detailed to be useful as an introductory text. The standard of English leaves much to be desired in places but this can hardly be blamed on the author, who should have been better served by his publisher. □

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News from the petroleum geologists

J.R.V. Brooks

Petroleum Geology of the Continental Shelf of North-West Europe, 1981. Edited by L.V. Illing and G.D. Hobson. Pp.521. ISBN 0-85501-656-6. (Heyden & Son: 1981.) £47, \$113.

It is now some 16 years since the first discovery of offshore gas in UK waters, and 13 years since the first commercial discovery of oil. In the period from 1964 to the present nearly 1,800 wells have been drilled.

To those involved in exploration and in the production of oil and gas these have been, and still are, exciting times filled with geological surprises. But to many geologists — nearly a generation of them — the scientific results of drilling have not been easily available, and it is increasingly apparent that universities — let alone schools — do not have the finances to purchase the data that now exist. Study of the geology of the UK and continental landmass of Western Europe gives only a partial picture of the total geology and geological history of the area. But when this is linked to what is now known about the North Sea Basins and other offshore areas, a more complete and meaningful picture emerges. From a teaching point of view, and to large numbers of geologists in non-oil areas, it is important that this new information becomes incorporated into the present state of knowledge.

Up-to-date data are hard to come by, but from time to time the industry holds a conference during which current views are aired and papers on exploration and proven fields published. The first conference on the petroleum geology of the continental shelf of North-West Europe was held in 1974, and the second in 1980 in London; it is the proceedings of the 1980 meeting which form the contents of the volume reviewed here.

The number (46) and range of papers presented is great; they are grouped into four main categories covering regional aspects of geology, technological developments, stratigraphy, and selected oil and gas fields. In some cases the opportunity has been taken to update material presented at the 1974 conference, but the majority of papers record more recent and current work, and present previously unpublished data.

Certain contributions were specially commissioned by the conference committee, notably those on temperature and thermal gradients in the North Sea, the regional seismic maps of the North Sea, and the subcrop map of the mid Mesozoic unconformity, the latter providing a particularly useful overview.

Clearly one cannot mention every single contribution and all of them are worthy of study, but there are a number of more significant papers that represent milestones

in exploration activity. Of these, the Brae field is as near as we have come to finding a stratigraphic accumulation of oil and its discovery has led to a search for further accumulations of this type. The discovery of the Frigg field is perhaps a unique example of a sedimentary unit which can be readily identified on a seismic section and might be described as a seismic/structural accumulation. In fact it is described as a preserved deep sea fan.

The largest discovery of oil onshore, made in 1973 at Wytch Farm, set off renewed interest in landward hydrocarbon exploration, which is currently being sustained. The results of this discovery have had considerable geological implications for exploration elsewhere in the UK, and the paper on Wytch Farm sets out the results and regional setting of the discovery.

Each of the papers in the volume represents recent results of exploration activity and might be considered as highlights of the book, published as they are for the first time. But the regional and field evaluations also serve to give the technical reader a wide overview of current hydrocarbon activity.

Such is the vast range and wealth of geology presented in this volume that no geologist, particularly those whose task it is to teach the subject, should be without a copy. Sadly, however, its cost may preclude the sort of distribution its contents deserve. □

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Greater clarity in lens biology

Christine Slingsby

Molecular and Cellular Biology of the Eye Lens. Edited by Hans Bloemendal. Pp.469. ISBN 0-471-05171-3. (Wiley: 1981.) £50.65, \$91.15.

PROGRESS in eye lens research, whilst resulting in a greater understanding of the organelle itself and its diseases, may also lead to discoveries in areas of more general interest. In his selection of ten contributions for this book, Professor Bloemendal has placed emphasis on the growing interest in cytoskeletal structures, as the role they play in controlling and maintaining cell shapes can be well observed in lens cells. To this end, the chapter by Lucio Benedetti *et al.* is

The heroine of this book is the lens itself: the molecular story of its refractile transparency is still untold. But by presenting the background to important questions, the book can be recommended to anyone who wishes to look for the answers. □

Peter D. Moore

There follows an unconformity in that the historical approach is reinstated for the detailed consideration of Australia's fossil flora and the development of phytogeographical regions. Again, one wonders whether a more natural sequence of topics could have been arranged. For example, Hélène Martin's detailed account of the Tertiary flora would have been better

This superbly produced set of books can be criticized only in that it has attempted to cover so vast a mass of information that arrangement and editing has evidently been a problem. Systematic and subject indexes are included, the former comprehensive, but the latter rather sketchy. These volumes establish a data base and reference source upon which biogeographical research in Australia will be built for many decades to come. □

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Anamorph anthology

C.T. Ingold

Biology of Conidial Fungi, Vols 1 and 2. Edited by Garry T. Cole and Bryce Kendrick. Vol.1, pp.486, ISBN 0-12-179501-2; Vol.2, pp.660, ISBN 0-12-179502-0. (Academic: 1981.) Vol.1 \$49, £32.40; Vol.2 \$68.50, £45.40.

AN alternative title for this work might have been *All About Anamorphs*, yet ten years ago this would have meant nothing to a mycologist. Now we accept a whole terminology of "morphs", much to the fore in *Biology of Conidial Fungi*. "Morph" is sometimes used in all its nakedness, but more usually as a termination: the teleomorph is associated with sex and meiosis; the anamorph with the asexual, conidial stage; and the holomorph is the whole fungus. The holomorph ideally is a teleomorph together with its anamorph, but it may consist of a teleomorph only, or even solely of an anamorph.

The present work deals with conidial fungi which are anamorphs of Ascomycetes or Basidiomycetes, together with forms in which the "perfect" stage is not yet known or has, perhaps, been lost in the course of evolution. The conidial types in the "lower" fungi, notably Mucorales, are not considered.

During the past decade or so Bryce Kendrick has devoted much of his abundant energy to promoting the study of conidial fungi, especially by his organization of the original Kananaskis workshop summarized in *Taxonomy of Fungi Imperfecti* (University of Toronto Press, 1971), and of the second in the same place devoted to the linkage of conidial anamorphs with their teleomorphs. This gave birth to the two volumes of *The Whole Fungus* (National Museums of Canada, 1979). Now, together with Garry Cole, a distinguished student of the fine structure of fungi, he has persuaded 35 authors from all over the world to cooperate in writing 31 articles on various aspects of conidial fungi.

The articles are grouped in seven sections: history; systematics; distribution and ecology; conidial fungi and man; ultrastructure, development, physiology and biochemistry; genetics; and techniques for investigation.

The first section has but one article, written by Bryce Kendrick, who also provides the initial chapter of the section on

systematics. One might, perhaps, expect these contributions to be a trifle dull; but not so. All that he writes throbs with enthusiasm and makes them a joy to read. The systematics section is one of the longest (260 pages), involving discussion of Coelomycetes, conidial yeasts, dimorphism, pleomorphism, conidial fungi growing on other fungi and lichens, and relations between conidial anamorphs and their teleomorphs. Nag Raj, writing about Coelomycetes, hands down ten exacting commandments for those rash enough to tackle these difficult organisms. Hawksworth contributes over 60 pages on fungicolous fungi. Although this is somewhat of an inflated list, it directs attention to these organisms which are much in need of detailed study.

In the ecological section, the chapters by Webster and Descals on fungi from freshwater habitats, and by Lacey on aerobiology, are particularly noteworthy and call for special commendation.

There is wide coverage of the economic importance of conidial fungi in connection with parasitism of crop plants, derma-

tophytes attacking man, mycotoxins, food spoilage and food technology, and the possibility of using certain species in the biological control of various parasites and pests. Although the fungi that prey on nematodes may not have much potential in such control, Barron's chapter on this subject is delightful.

Ultrastructure has a good innings with an illuminating chapter by Garry Cole. Nuclear behaviour is treated by Robinow in his usual masterly way. There is an interesting article by Lemke on fungal viruses and a splendid contribution by Aronson on hyphal walls.

The only chapter in the genetics section is by Hastie. This is outstanding, a real gift to mycologists at all levels.

The whole work is a major contribution to mycology and, although inevitably lacking some coherence, should find a place in all institutions where fungi are studied. □

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Beyond mechanics in human reproduction

R.V. Short

Conception in the Human Female. By Robert G. Edwards. Pp.1,087. ISBN 0-12-232450-1. (Academic: 1980.) £48.50, \$116.50.

Dr Edwards is to be congratulated on having produced what may well turn out to be the most influential text in the field of obstetrics and gynaecology published this century. Hitherto, these specialities have been surgically orientated, and principally concerned with the mechanical difficulties of delivering babies, repairing prolapses and treating gynaecological cancers. This they have performed well, as attested in part by the maternal and perinatal mortality statistics.

Nonetheless the time has come for the profession to rise to a new challenge. Advances in our understanding of endocrinology, infertility, contraception and gamete biology have overtaken the surgical aspects of the subject, and one hopes this textbook will serve as a basis for the training of the obstetrician/gynaecologist of the future. He or she now needs to know more about endocrinology than those in general medicine, more about male reproduction than the urologist, and needs to develop a new understanding of genetics, reproductive biology, embryology, immunology and human sexuality. It is no accident that this excellent book was written by somebody who is not medically qualified; it is time for obstetricians and gynaecologists to rise to the challenge and develop their scientific understanding in parallel with their manual skills.

Dr Edwards has obviously chosen to discuss those topics that have been of greatest concern to him in the past 18 years in Cambridge, where in collaboration with Patrick Steptoe he pioneered the technique of human *in vitro* fertilization against open opposition from Nobel Laureates and masterly inactivity on the part of the Research Councils. Each chapter in the book is extensively referenced, and absolutely up-to-date at the time of going to press in early 1980. It is also particularly well illustrated, using key graphs, tables, diagrams and photographs taken from the work of others, duly acknowledged. The fact that Dr Edwards has been a stimulating teacher of undergraduates in Cambridge is reflected in the text — he knows what to discuss, and how to present it in the most exciting and informative manner.

It will be disappointing if the word "Human" in the title means that this book will be restricted to medical libraries, and the bookshelves of consultants who are able to afford it. It deserves a far wider readership. Although principally concerned with human beings, Edwards takes a broad approach, and is not inhibited from discussing the wider comparative aspects of the subject. The book is destined to become a "must" for anybody working in the general field of reproductive biology — a treasured possession. For it really is excellent. □

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Macmillan Reference Books, London (in North America, Facts on File) have recently published *The R.A.E. Table of Earth Satellites, 1957-1980*, compiled by D.G. King-Hele *et al.* at the Royal Aircraft Establishment. The *Table* contains a chronological list of 2,145 launches and mention of some 12,000 satellites and associated fragments, details being given as appropriate. Price is £30.

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